



US health politics may give insurers a respite on genetic information

Washington. The need to preserve the fragile political consensus behind a health reform bill in the US Senate appears to have dissuaded one of its leading supporters from backing an amendment barring health insurance companies from using genetic information to deny coverage to potential clients.

Nancy Kassebaum (Republican, Kansas), the chair of the Senate Labor and Human Resources Committee, said last week that she was not planning to amend the Health Insurance Reform Act to include the words "genetic information" in a list of factors insurers may not use to exclude people.

This is despite the urging of scientists such as Francis Collins, the director of the National Center for Human Genome Research (NCHGR), who are concerned that uncertainty about insurance prospects is already making some individuals reluctant to participate in screening projects.

The bill, sponsored by Kassebaum and Edward Kennedy (Democrat, Massachusetts), guarantees continued insurance coverage to those who become ill or change or leave their jobs. To this end, it bars insurers from denying insurance on the basis of factors that include pre-existing medical conditions and medical history.

Although such factors do not explicitly include 'genetic information', the so-called 'report language' accompanying the bill — a plain-English synopsis intended to clarify arcane legislative language — asserts that the term 'medical history' should be read to include genetic information.

Kassebaum says that this informal language is sufficient protection. "I doubt that we would go beyond that," she said. But the informal language is not legally binding, and the concern of scientists has been expressed through Tom Harkin, (Democrat, Iowa), who tried unsuccessfully to add genetic information to the bill in committee.

Observers say Kassebaum's resistance has political roots. "The [whole] bill hangs by a very fragile majority," says Michael Tanner, a health policy analyst at the Cato Institute, a libertarian think-tank. If the bill is opened up for amendments, a "flood of proposals", both liberal and conservative, could end its prospects, he says.

Researchers are concerned that if no explicit reference is made to genetic information in the bill, individuals may be reluctant to participate in trials involving genetic testing on the grounds that this might prejudice their chances of obtaining insurance.

Indeed, some claim that this is already happening. "People certainly at least think

twice about whether they want to participate," when they realize their insurance may be threatened, says Elizabeth Thomson of NCHGR, who is overseeing a multi-centre trial of genetic testing and counselling for people at risk of heritable breast, ovarian and colon cancer.

But insurers insist that they must be allowed to deny coverage to individuals who may be at high risk because they carry disease susceptibility genes. Otherwise, they argue, people who are poor health risks, but who do not reveal this fact, will raise the costs of individual policies for everyone.

"We don't think it's basically fair," says Harvie Raymond, an assistant vice president at the Health Insurance Association of America, adding that it is not "appropriate" for the "viability and profitability" of insurance companies. He adds that fears about insurers using genetic information to exclude people are unwarranted, given the existing restrictions in the Kassebaum-Kennedy bill.

But Collins, who co-chaired a working group last year whose report proposed both state and federal legislators should move to prevent genetic discrimination in health insurance, disagrees. He urged Kassebaum,

at a hearing of the Senate committee last week, to include the words 'genetic information' explicitly in the bill. "If you limit this category to medical history, you're going to miss some people," he said.

Collins argued, for example, that under the bill as drafted, insurers might be able to deny coverage to individuals who were shown by genetic tests to carry genes for recessive disorders such as cystic fibrosis.

Also affected, said Collins, could be women such as those in a recent study of *BRCA-1* mutations associated with early-onset breast cancer. Nearly half of such women with mutations had no family history of the disease, as the mutation had been carried asymptotically by their fathers, Collins said.

The Kassebaum-Kennedy bill is expected to reach the Senate floor in the third week of April. As written, it has strong backing, with 51 out of 100 senators agreeing to act as co-sponsors. A Republican initiative in the House of Representatives which is intended to match the Senate bill contains no reference to genetic information. It is expected to reach the House floor in the last week of March.

Meredith Wadman

See also report on page 96.

Hubble brings Pluto's surface into view

Washington. The first detailed images of the surface of the planet Pluto, taken by the Hubble Space Telescope in 1994 and unveiled at a press conference last week, show a world with more large-scale contrast than any planet in the Solar System except Earth.

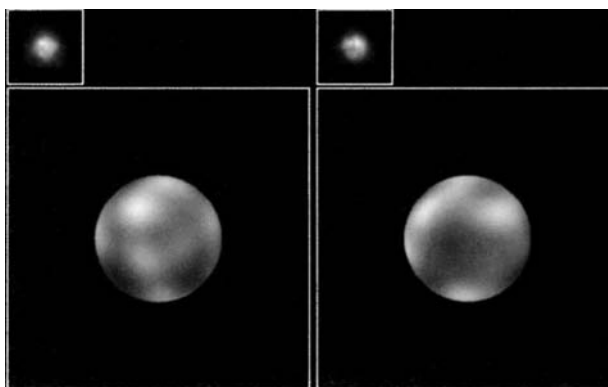
Up to a dozen distinct albedo features — dark and light provinces — appear in the pictures (above right), which are still being analysed by Alan Stern of the Southwest Research Institute in Boulder, Colorado, Marc Buie of the Lowell Observatory in Flagstaff, Arizona, and Laurence Trafton of the University of Texas at Austin.

The large images shown here are reprojections based on surface maps

created from the original Hubble images, which are shown as insets.

The images, using photographs taken with the European Space Agency's Faint Object Camera, show bright polar caps (believed to be areas of nitrogen frost), a bright equatorial spot that rotates with the planet, and several 'dark' regions (probably where sunlight has broken down surface hydrocarbons).

Tony Reichhardt



NASA/ESA

Canadian technology budget downgrades basic science

Montreal. A new budget announced last week by Canada's Finance Minister, Paul Martin, has produced plans for a mechanism to help companies compete internationally in high-technology industries.

But the proposals seem certain to confirm the fears of many basic scientists that they are not among the Liberal government's priorities, thus further eroding what researchers see as the basis of the country's technological advances.

Furthermore, the new approach, called Technology Partnerships Canada (TPC), will involve no additional money, but will rely on loans using funds reallocated from existing government programmes.

The budget also includes a cut of more than 10 per cent in federal financing for Atomic Energy of Canada Limited. This will mean dropping all research not connected to the Candu nuclear reactor, including projects such as the neutron-scattering laboratory, nuclear fusion work and support for the Sudbury Neutrino Observatory.

TPC forms the centrepiece of Canada's long-awaited science and technology strategy, *Science and Technology for the New Century* (see *Nature* 377, 665; 1995). First promised early last year, the strategy is the outcome of nationwide consultations and focuses in particular on the need to develop partnerships between the private sector, academic institutions and other governments.

The strategy will involve what the government calls "a new system of governance". This will include the creation of a Science and Technology Advisory Council to advise the prime minister and cabinet, and the requirement that all federal departments and agencies report annually to parliament on their expenditures, activities and priorities in science and technology.

Despite the apparent logic of such moves — which are broadly similar, for example, to those introduced by the British government three years ago — some sceptics are already pointing out that the advice of federal advisers and advisory bodies on science and technology in Canada is often ignored.

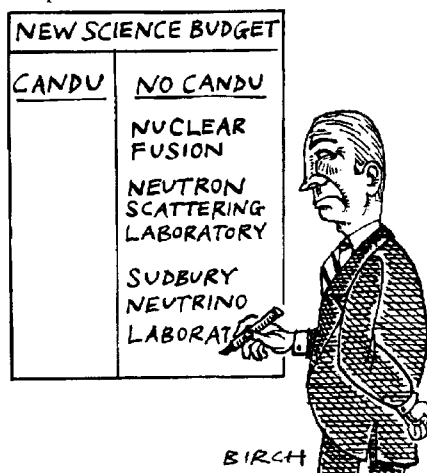
It is not long, for example, since a body set up by the federal government to provide it with advice on science policy — the Science Council of Canada — was abolished, and the recommendations of the prime minister's National Advisory Board of Science and Technology were rejected.

The TPC will involve what the government calls "investments" amounting to C\$250 million (US\$183 million) a year by 1998–99 that will be fully repayable. The government will share with the private sector both risks and rewards: money from the fund will be repaid from royalties generated

by successful projects, and will be recycled back into the fund for future investments.

Potential partners in TPC will be "well-managed, financially-sound incorporated businesses that can demonstrate their ability to successfully complete their project", says Industry Canada, the government agency responsible for the programme. These can include groups or alliances, associations or consortia from all regions of Canada.

Government officials say that although Canada has a strong tradition of achievements in fields such as telecommunications, medicine and software, its overall performance lags behind that of its international competitors. This has contributed over the



past two decades to weak productivity, sub-optimal employment growth and large government deficits.

The fund's focus will be on environmental technologies, on what are called "enabling technologies" such as biotechnology, information and advanced manufacturing technologies that make industries more efficient and productive, and on the aerospace and defence industries, including "defence conversion".

Facilities slated for closure in three months time under the AECL budget cuts include the neutron-scattering laboratory at Chalk River. Materials testing methods developed there demonstrated the integrity of the sealant ring that was redesigned after a similar one led to the explosion on the space shuttle *Challenger* in 1986.

Federal support for fusion research, which is overseen by AECL, will also cease in a year's time. The director of the Canadian Centre for Magnetic Fusion in Varennes, Quebec, which operates the fusion reactor, says he expects this will mean the end of its programme, although he plans to fight the cuts. The Canadian Fusion Fuels Technology Project in Toronto is also expected to close.

David Spurgeon

Courts back research and compensation for Japanese HIV victims

Tokyo. A legal battle between HIV-positive haemophiliacs and five companies that distributed contaminated blood products moved closer to resolution last week, when the Osaka and Tokyo district courts released a new compromise proposal, a revised form of a compromise suggested last October (see *Nature* 377, 467; 1995).

In addition to financial compensation, the courts recommend that the government should set up a medical institution to provide treatment to AIDS and HIV-positive patients, to carry out research into AIDS and to provide education to other hospitals about AIDS and treatments for HIV.

The new plan recommends that, in addition to the ¥45 million (US\$430,000) compensation for each plaintiff recommended in the initial compromise, the defendants should also set up a system for providing medical treatment and financial assistance to the victims. This proposal is probably the last to be offered by the courts, and is widely expected to bring the six-and-a-half year-old court case to a conclusion.

The courts' proposal confirms that the defendants are responsible for the disaster, in which an estimated two-fifths of Japan's 5,000 haemophiliacs were infected with HIV. It recommends that they pay ¥150,000 a month to each patient who has subsequently developed AIDS.

Testu Noma, the counsel for the plaintiffs in the Tokyo court case, says the proposal does not meet all the group's demands for compensation. But he is optimistic that an agreement can be reached, provided that the companies can be persuaded to apologize.

Japan's new minister for health, Naoto Kan, recently apologized to the victims and admitted the government's responsibility for its role in the continued use of blood products which had not been heat-treated to kill HIV and other viruses (see *Nature* 379, 663; 1996). But the five companies that distributed the contaminated blood products have remained reluctant to admit negligence.

Two foreign-based companies in particular — Baxter Ltd and Bayer Yakuin Ltd — have been loath to make admissions of guilt, possibly because such admissions might be used against them in other countries where the companies operate.

The plaintiffs plan to visit the offices of each of the five companies this week to receive apologies before the deadline for the compromise proposal expires at the end of this month. If no agreement is reached by then, says Noma, the matter will revert to the courts, and justice, he says, may be delayed indefinitely.

Stephen Barker

US plans for virtual laboratories by Internet

THE US National Science Foundation (NSF) is to launch a project to create a high-speed computer network that will allow US scientists to carry out activities — such as collaborating in virtual reality laboratories and manipulating instruments by remote control — that are impossible to do properly on the existing Internet.

NSF also hopes that the programme, known as 'Connections to the Internet', will encourage the development of technologies that would allow its planned high-speed network to be grafted onto the existing network.

Since last year, NSF has been leasing a 155-megabit very high-speed Backbone Network Service (vBNS) from the telephone company MCI. Until now, the vBNS has linked only NSF's supercomputers (see diagram) and has had few connections to the Internet.

In future, however, NSF will ask researchers to submit proposals on ways of using the backbone for high-speed networking. It will also fund high-speed connections from their home campuses to the vBNS, according to Mark Luker, director of both the vBNS and the new programme.

The immediate impact of such grants will be to allow scientists at a few dozen universities to interact at high speeds for the first time. Researchers will be able to operate instruments such as telescopes by remote control, and collaborate using virtual reality laboratories.

But the prospect of gaining access to the vBNS is also a 'carrot' which NSF hopes will eventually encourage universities to invest their own money in upgrading local networks, and in doing so, create the embryo of a national high-speed network.

Building such a network is the only way to bring down the costs of high-speed networking. Leasing a direct high-speed link connecting one university to another can cost as much as \$500,000 a year, according to Thomas DeFanti, director of the Electronic Visualization Laboratory at the University of Illinois in Chicago. In contrast, a 155-megabit link to the vBNS backbone, which in turn would connect to many other campuses, costs only \$66,000 a year.

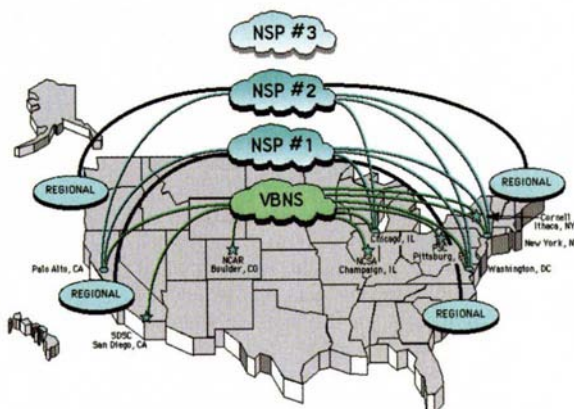
With some clever engineering, some high-performance tasks can already be carried out through the Internet. But campus Internet links are becoming congested as students flock to use the World Wide Web (WWW), which uses up much more bandwidth than either e-mail or file transfer.

As a result, many researchers feel that high-performance computing has become almost impossible, while the transfer of large data files often takes a long time.

But according to Luker, the need for

high-speed networking is due less to the problems of congestion than to the wish of researchers to develop new ways of collaborating with each other. "We have people who want to make more sophisticated use of networks, but they are stymied," he says. "They have no way to do it short of leasing their own lines."

The Internet was not designed for high-speed applications, which typically require not only high bandwidth but also close synchronization between computers. Instead, it was planned primarily to make the most effi-



The National Science Foundation's Internet 'backbone' following privatization last year. NSF now funds only the high-speed vBNS linking its five supercomputer centres (☆). The rest of the backbone is run by commercial, regional and national network service providers (NSPs).

cient use of telephone lines, by smoothing out over both time and space the uneven stream of data packets from applications such as e-mail, file transfer and the WWW.

In particular, the design of Internet means that its response times are highly variable. Operating a telescope by remote control using Internet can be like driving a car whose response to the steering wheel may be ten seconds, unknown, or vary from one second to the next, says DeFanti.

Building a separate high-speed network for researchers does not solve the problem, says Luker, who claims it would defeat a general goal of sharing network resources, and be impossible to scale up economically. NSF's vision, he says, is "a single Internet that supports all services".

But neither would simply linking the two networks together be a solution, as the high-speed network would be quickly invaded by hordes of web-browsing packets. A technical 'fix' is first needed that would either give priority on the hybrid Internet to high-performance applications or code such traffic so that it is diverted to the high-speed backbones. (At present, the Internet treats all packets alike).

Stimulating the development of this technical fix is one goal of NSF's new programme. The agency will not merely provide researchers with a direct line to the vBNS,

but will also ask universities to upgrade their networks to handle both traditional Internet traffic and traffic destined for the vBNS. This should be done in a way that can be scaled up economically to the dimensions of the Internet, adds Luker.

There is no shortage of ideas about how this prioritization could be achieved. One way would be to reprogramme 'routers' on the network to sort data packets according to the address of the sender, as packets from known high-speed computer users would be automatically routed to the vBNS.

NSF hopes that whatever technical solution emerges, the end result will be an improved service, which Luker points out cannot currently be bought on the Internet. According to Luker, NSF is introducing the concept of prioritization to Internet traffic, with users paying first- or third-class, depending on the service they want.

NSF's move has some familiar aspects. The agency catalysed the early growth of the Internet in the 1980s by financing a national 'backbone' to which campuses could connect.

Similarly, NSF funding for upgrading campus networks to carry high-speed prioritized traffic could eventually lead to the creation of a national high-speed network.

Nonetheless, the early growing pains of 'Internet II' are likely to be much greater than those of the current network. The circumstances are very different. The Internet originally grew out of university and other publicly supported networks; by the time providing Internet connections was transferred to commercial operators, it had become what economists refer to as a 'commodity business', and prices were quickly driven down by fierce competition.

This time the situation is reversed. The three US national high-speed 'backbone' networks are owned by telephone companies — MCI, Sprint and AT&T — while the extensive cabling in urban areas is also owned by private companies.

The current high costs of high-speed networking mean that services such as on-line video are not yet economic. With few profits to be made from an embryonic network, the commercial companies involved are reluctant to connect with each other, preferring to hold out and fight for market dominance. Costs will eventually collapse, predicts DeFanti, "but not for several years".

Indeed, while Internet I was born in America, 'Internet II' may originate in Europe, where many countries are nearing completion of national high-speed research networks. Such state-owned networks may be easier to link up, says DeFanti, adding that "there is less of the need for a business case at every turn".

Declan Butler

Transgenic trials under pressure in Germany

Munich. Germany's largest environmental protection pressure group, BUND, has called for a ban on all field experiments with genetically-manipulated plants, following the publication of a report on experiments demonstrating the spontaneous transfer of a gene from a transgenic oilseed rape hybrid into a closely related weed-like plant.

The reaction to the results has put further pressure on German plant geneticists, who already experience a level of hostility to their field experiments not encountered in other countries. As a result, relatively little research in molecular plant genetics is carried out in Germany compared with other large European countries.

Scientists in Germany, as well as the authors of the paper published last week in *Nature* as an item of Scientific Correspondence (see *Nature* 380, 31; 1996), claim that environmentalists are deliberately misinterpreting the data to 'prove' a danger that the scientists deny exists.

The transgenic crop in question, *Brassica napus*, was modified to carry the gene which encodes the enzyme phosphinothricine acyl-transferase, so conferring resistance to the herbicide glufosinate which is sold in Europe under the name BASTA. The patent on the gene is owned by the German company AgrEvo.

The report shows that within a couple of growing seasons, the herbicide-resistant gene was able to transfer into *B. campestris*, the weed-like plant. The authors of the paper first crossed the oilseed rape carrying the herbicide tolerant gene with *B. campestris*. They then crossed these hybrids back with *B. campestris*. These new plants, which were stable and fertile, were found to contain the tolerance gene.

Thomas Mikkelsen, a research student at the Risø National Laboratory in Denmark,

who co-authored the report, says that such spontaneous gene transfer cannot be extrapolated to other transgenic crops, such as potatoes or maize, as they do not have close weedy relatives in Europe. In addition, crossbred plants, like crossbred animals such as the mule, are usually sterile.

But this has not stopped environmentalists from exploiting the publicity the report has prompted, with headlines claiming that 'superweeds' could soon overrun food crops. Senta Seip, a spokesperson for the Greens in Hessen, for example, said the results show "the risks of gene technology cannot be predicted", and "confirm the party's view that field trials with gene-manipulated rape should be abandoned".

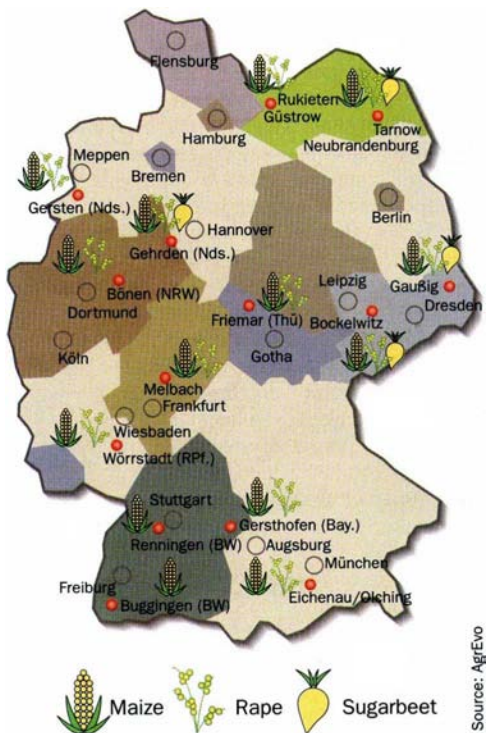
Last year, all 15 small field trials conducted by universities and research institutes in Germany were partially or fully destroyed by activists, even though most were studying the environmental safety of growing genetically-manipulated plants in normal agricultural environments. Larger scale industrial field trials were also attacked, but suffered relatively fewer problems because the size of the fields makes them more difficult to destroy.

Gerhard Wenzel, a professor at the Technical University of Munich, runs a project funded by the Bavarian government's Forbiosich (safety in biotechnological research) programme to study the spread of the glufosinate gene from transgenic maize and rape through soil and via airborne pollen. Frustrated by repeated attacks on his crops, Wenzel wants the authorities to construct a fence around his field site south of Munich. But this has always been rejected, he says, on the grounds that it could disturb wildlife.

At the same time, Wenzel acknowledges that a security fence could provide environmental activists with ammunition for claims that transgenic crops are dangerous. Gerhard Waitz, a spokesman for AgrEvo, which last year lost two trials with glufosinate-resistant crops to activist attacks, says the company made a deliberate decision not to embark on security exercises. "If you want to sell a product, you cannot start by putting it behind fences," he says.

Other solutions are expensive. Detlef Barsch, a postdoctoral fellow from the University of Aachen who is heading a project on the environmental impact of virus-resistant sugar beet, was attacked with stones last year when trying to evict activists from a field site near the Dutch border. He used part of his grant money from the federal research ministry to pay for a nightwatchman to guard his crops until harvest.

Scientists have also considered carrying out their field trials in other countries where



Some sites of open-air experiments with genetically modified crops in Germany.

they are less likely to be attacked. But this has not proved feasible for various reasons. Lothar Willmitzer, director of the Max Planck Institute for Molecular Plant Physiology in Golm, near Potsdam, east Germany, says plant geneticists should not abandon their rights to carry out field trials in Germany because of pressure from activists. On a more practical level, he points out, scientists need to be physically near their trials.

But Barsch, who fears his scientific career may suffer if he cannot complete his work, hopes to conduct his 1997 trial in the Netherlands, provided he can obtain a grant from the European Commission; his current grant does not cover trials abroad.

Despite such caution, the pressure group Genetisches Netzwerk launched a campaign last week based on claims that scientists are trying to exploit the situation in east Germany, where economic concerns outweigh radical environmental concerns, by turning more frequently to conducting trials there.

Henning Strodthoff, a spokesman for the group, says that "the choice of sites with the lowest potential for protest shows that [scientists] feel they can exploit the economic difficulties of people".

Most of the applications are from commercial companies needing to show that their test crops can be grown safely in local conditions in order to market the crops in Germany in the future, and have been encouraged by last year's relaxation of genetic release laws, reducing the bureaucratic burden of preparing trials. **Alison Abbott**

Europe 'sliding back' in technology

London. A European Commission annual survey of industry has warned that the European Union (EU)'s declining share of patents — compared to Japan and the United States — could threaten its commercial competitiveness in fields where the EU currently enjoys "technological leadership".

The annual *Panorama of Industry*, published at the end of February, says the EU lost patent share "in all sectors" between 1984 and 1993 except aerospace and transport equipment. "The most worrying observation", says the report, "is the rapid further deterioration of the EU's already weak position in the electronics sectors", where research and development activity "is at its most intense at the world level". □

Lobbying in US Senate heads off restrictions on foreign researchers

Washington. An immigration bill scheduled for debate in the US Senate next week has been amended to remove barriers to hiring non-US citizens that were contained in an earlier draft. The change, announced last week, makes it unlikely that laws regarding employment of legal immigrants — including researchers — will be changed substantially this year.

The decision to accept the amendment follows intense lobbying by a coalition of businesses, universities, civil rights groups and other organizations over the past few months against the employment provision in the bill, sponsored by Senator Alan Simpson (Republican, Wyoming).

Simpson had originally proposed a sharp reduction in the number of foreign-born professional workers allowed into the country (see *Nature*, 378, 117; 1995). His bill also called for a two-year waiting period before non-US graduate students could be hired by a US employer. It eliminated the category of 'outstanding researcher' under which scientists and engineers could be exempted from a long certification process, and imposed a fee on employers who wanted to hire non-Americans.

All these provisions have now been stripped from the proposed legislation. A frustrated Simpson, acknowledging the intense pressure on the committee from business interests such as the computer company Microsoft, which want to retain the option of employing foreign-born programmers, said last week that he would remove "every shred" of employment-based restrictions from his bill.

Lamar Smith (Republican, Texas) has already made similar concessions in amending his complementary immigration bill in the House of Representatives, which will be merged with the Senate bill before coming to a vote, probably within the next couple of months.

Although immigration has surfaced as a potentially volatile issue in the current presidential campaign, it cuts across party political lines, with Democrats and Republicans taking part on both sides of the debate. Almost three-quarters of Americans said in a recent poll that they favour tighter immigration laws. But businesses and universities have been especially vocal in protesting against additional restrictions on hiring foreign-born talent.

A recent meeting between Simpson and the chief executives of 50 US companies failed to defuse this opposition, which has thus left him no choice but to remove the planned restrictions, according to Senate staff.

Tony Reichhardt

Chemists sound warning on sponsorship of exhibitions

Washington. The American Chemical Society (ACS) has broken off talks with the Smithsonian Institution about revising the *Science in American Life* exhibition, ending the hopes of critics that the controversial show would be revised with a more 'pro-science' perspective.

In an article in this week's issue of the ACS publication *Chemical and Engineering News*, the society will go public with its criticisms of the exhibition for the first time. It will also warn other potential donors against sponsoring exhibitions at the Smithsonian if they want to retain any control over the result.

The ACS pulled out of the talks — which were initiated a year ago by Michael Heyman, the secretary of the Smithsonian — after concluding that they were not going to lead to the changes it was seeking.

According to ACS officials, the last straw came in December, when the Smithsonian offered the society a list of 35 changes it would make, and asked for \$400,000 to make them, on top of the \$5.3 million that the ACS had already paid to mount the exhibition. The ACS rejected the offer and abandoned the talks.

The *Science in American Life* exhibition has come under criticism from scientists and others because of its emphasis on arguments about intellectual property rights and its stark portrayal of the development of the atomic bomb. Its defenders argue that the exhibition breaks new ground by assessing science's relationship with society, rather than simply trumpeting its achievements.

"We've been working with the Smithsonian at great length, and we feel we've reached an impasse," says Joan Shields, a professor of chemistry at the Long Island University, New York, and chairwoman of the ACS. "Every time we meet with the directors of the Smithsonian, they seem to agree that change is needed, but either they don't know how to do it, or they won't do it."

Shields says that the ACS had been working consistently behind the scenes to change the exhibition, both before and after its opening in April 1994, and was only now going public about its concerns. The exhibition, she says, is "a litany of revisionist history" which draws attention to "social injustice and environmental catastrophe" without balancing them against the achievements of science.

The exhibition will continue to run permanently at the National Museum of American History on the Mall in Wash-

ington DC, with sponsorship attributed to the ACS. No changes to its original content are expected. "We are out of the picture now," says Shields. "It is unlikely that the Smithsonian will do anything, now that we're not knocking on their door."

Shields added that she thought the society's 150,000 members would understand both its original decision to spend money on the exhibition while ceding control over its contents, and its recent decision to stop negotiations over changes. "We had a goal in mind," she says, including better public understanding of science, and attracting young people into careers in science and

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The chemistry of history: American Chemical Society has criticized the *Science in American Life* exhibition.

chemistry. "We thought it would be achieved, but it hasn't been."

The American Physical Society (APS) — which had no role in sponsoring the exhibition — publicly attacked it soon after its opening, leading Heyman to promise "some changes which will render the exhibition a lot more balanced" (see *Nature* 374, 207; 1995). But Bob Park, head of public affairs at APS, said that nothing had since been done to the exhibition. The matter is expected to be raised by the APS Council at its general meeting in St Louis, Missouri, next week.

The Smithsonian has produced its own hundred-page assessment of the exhibition, based on a survey of 400 visitors. It found that "the visiting public entered the exhibition with a very positive view of science and technology, and that their views were reinforced and confirmed, rather than changed in either a positive or negative direction".

The ACS provided the funding for *Science in American Life*. But, as the sponsor, it had no more editorial control over the selection of the material exhibited than any other private company sponsoring a cultural event. In February last year the Smithsonian promised to consult the visiting public, the ACS and other interested parties before implementing changes to the exhibition.

Colin Macilwain

Genome ethics chair resigns amid worries over autonomy

San Francisco. Lori Andrews, the chair of a working group set up jointly by the US National Institutes of Health (NIH) and the Department of Energy (DOE) to provide advice on the ethical, legal and social implications of the Human Genome Project, has resigned, citing concern about the autonomy of the group.

The working group was set up to advise Congress, President Bill Clinton and the public on the implications of the \$3-billion gene-sequencing project. It is funded through the National Center for Human Genome Research (NCHGR), although separately from the 5 per cent which is set aside for so-called ELSI (ethical, legal and social implications) research grants.

Various members of the working group have recently been expressing frustration about interference by NCHGR. Their main complaint is that, over the past year or so, the centre has become increasingly involved in the working group's budget allocation and position statements.

At the centre of the dispute, they claim, is the working group's responsibility for representing a range of diverse interests — from geneticists to consumer advocates — which can conflict with the more focused concerns of scientists engaged in mapping and sequencing the human genome.

At a meeting with Francis Collins, director of the NCHGR, on 25 January, the executive committee of the working group urged him to reduce the involvement of his staff in assessing the working group's activities and in representing ELSI policy interests in Washington. But Collins — who was not available for comment this week — is said to have proposed merely that NCHGR should deal with day-to-day policy matters while the working group addresses longer-term issues.

Some observers suggest that Collins may have been reacting to perceptions that the working group has had difficulty in persuading legislators to enact policies related to the human genome. New measures on issues such as privacy and discrimination could be critical both to the genome project's long-term viability and to its public acceptability.

David Cox, co-director of the Stanford Human Genome Center, and a member of the working group, says that both NCHGR and the working group should be able to pursue policy issues. But he says that because of its composition, the group may have to move more cautiously than NCHGR staff.

Paul Billings, acting associate professor of medicine at Stanford University, says that the ELSI working group has already proved its effectiveness in cases such as a suit filed recently against the Department of

Defense's DNA Specimen Repository (see *Nature* 379, 385; 1996). In that case, ELSI-funded research provided the ammunition for negotiations about procedures for obtaining proper consent, confidentiality and other restrictions on genetic information storage.

The immediate trigger for the disagreement leading to the departure of Andrews, who is professor of law at the University of Chicago, appears to have been concern among working group members about behavioural genetics. The group had planned to spend \$20,000 on developing an anthology on the "non-medical uses of genetic information", later renamed as a collection of papers on behavioural genetics.

But NCHGR told the working group that such a project would be too expensive. When the group prepared a statement last year on the arguments linking race and intelligence put forward by Richard Herrnstein and Charles Murray in their book, *The Bell Curve*, it was held up for eight months while it went through a series of reviews (see *Nature* 378, 529; 1995).

Troy Duster, professor of sociology at the University of California, Berkeley, and vice-chair of the working group, says that the call for papers for the planned collection may have been squashed partly because of concern among geneticists working on behavioural issues that the working group would put together a general attack on their area of research.

In a memorandum sent last week to committee members, Duster wrote that, as a member of the National Advisory Council for Human Genome Research, he had found it "impossible to reconcile '\$12 million to this lab' and '\$8 million to that lab' with the working group's possible allotment of \$25,000", adding that this was "transparently insufficient funding to pursue the ethical, legal and social issues of the non-medical uses of genetic information". In addition, NCHGR cut the working group's budget by 5 per cent in the current fiscal year, and limited its activities to one ELSI meeting.

Andrews' resignation came as the working group's task force on genetic testing prepares to release for public comment draft principles for the delivery of genetic tests. Duster, who has stepped in as Andrews' temporary replacement, says that he has no interest in chairing the working group in the current circumstances. He adds that decision-making authority over its overall budget, its allocations of grants, working group projects and group policy statements, must be clarified. "We have to be autonomous," says Duster.

Sally Lehrman

Pollution inspectors 'inconsistent' about radioactive material

London. The British government's radioactive waste management advisory committee (RWMAC) has criticized its pollution inspectorate for adopting a rigid — and sometimes inconsistent — attitude to the use, storage and disposal of small amounts of radioactive materials. This has led to a 12-fold increase in prosecutions in the space of a year, the committee suggests.

The number of prosecutions of these "small users" of radioactive materials, mainly universities and hospitals, pursued by Her Majesty's Inspectorate of Pollution (HMIP) increased from one a year between 1989 and 1991, to 12 in the year 1991–92. Fifteen users were prosecuted in 1994–95.

In a report on the problems encountered by such users, RWMAC, a 20-member group chaired by Sir Gordon Beveridge, vice-chancellor of Queen's University, Belfast, said it found evidence of inconsistencies in the way safety regulations were enforced. It doubted that the sharp rise in prosecutions was entirely the fault of operators. "A decision to prosecute more readily seems the most likely explanation."

The advisory committee, in a chapter entitled "Attitude of inspectors: diplomatic relations restored?", criticizes the inspectorate for sending inexperienced and inadequately trained staff. It also cites complaints from users who said inspectors tended to be uncooperative and unwilling to dispense advice. It calls for the reinstatement of specialist inspectors, the setting up of a users' help desk, and adoption by HMIP of a more "pragmatic approach" towards resolving problems.

The report cites several examples of inconsistencies in enforcement. In one case, a small user was prosecuted for inadvertently disposing of small amounts of radioactive clinical waste in an appropriate incinerator, but one he was not authorized to use. But another user who disposed of radioactive waste in an incinerator that was not authorized for such disposal received only an enforcement notice. The committee says it is essential for an enforcement action to relate to the level of offence. "Otherwise a perverse incentive might be created to cover up rather than to own up."

The report acknowledges "evidence of at least some relaxation in the attitude and approach" of the inspectorate towards understanding the problems of small users. But it adds that many problems still remain. These include a lack of appropriate sites where small users can accumulate, store and dispose of radioactive waste, insufficient incinerators and landfill sites, excessive paperwork, and high administrative charges.

Ehsan Masood

Meeting warms to binding climate agreement

London. An international law penalizing countries that fail to meet agreed targets for reducing greenhouse gas emissions edged a step closer to reality last week after a meeting in Geneva of representatives of countries that have signed the climate change convention.

The United Nations Framework Convention on Climate Change, agreed at the Earth Summit in Rio de Janeiro in 1992, recommends — but does not legally bind — developed countries to reduce greenhouse gas emissions to 1990 levels by 2000.

Although some countries have already begun to implement policies designed to achieve this goal, many others, including some of the largest emitters such as the United States, have made little progress.

Now many signatories to the convention — including the United States, China and the European Union — have agreed on the need for a legal instrument to ensure that countries comply with future targets to reduce emissions of greenhouse gases.

Both the proposed new agreement and the question of specific targets for emission reductions are expected to be finalized by the third annual conference of the climate convention to be held, possibly in Tokyo (see below), late next year.

There are two broad options for the new law. The European Union and the Alliance of Small Island States (AoSIS) are backing a protocol — a legally binding treaty — to regulate greenhouse gas emissions that would be independent of the climate convention.

Such an approach would mirror that of the Montreal Protocol banning ozone depleting substances, often cited as an example of a successful protocol that broke off from its 'mother' convention — the Vienna Convention on the protection of the ozone layer.

But this approach is not unanimously favoured. US delegates in particular, while stating that "the United States is not

opposed to a protocol *per se*", emphasized the advantages of amending the existing climate convention rather than enacting a new treaty requiring fresh signatures and a new set of associated institutions.

Whatever form the new legal instrument takes, some countries, such as Japan and Canada, warned last week that it was premature to discuss the choice of an instrument without first outlining its substance.

AoSIS — some of whose states risk severe flooding if sea levels continue to rise — want a protocol that binds countries to reduce greenhouse gas emissions by 20 per cent by the year 2005. Russia, in contrast, while agreeing on the need for a protocol, argues that targets for emission reductions should reflect a country's economic health.

The outcome of the debate will also be directly affected by the absence within the climate convention of an agreed decision-making procedure for a protocol.

The enactment of any legal instrument would have to pass through two stages — adoption by member countries meeting at a climate conference, followed by ratification in national parliaments.

An amendment to the climate convention needs ratification by three-quarters of the member countries. A protocol, however, would be more difficult to secure, as the convention does not set out rules for adopting or ratifying a protocol. In the absence of such rules, a decision on a protocol will — like other issues in the climate convention — need the consent of all parties.

Forging any such consensus, however, is controversial, particularly, among environmentalists. The Climate Action Network (CAN), an alliance of groups that includes Greenpeace, the World Wide Fund for Nature and the Natural Resources Defense Council, would like member countries to resort to majority voting, as this would prevent small, vocal minorities from blocking the implementation of particular measures.

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Clearing the way? A new climate protocol would require extensive groundwork.

"When consensus fails, the parties must have some mechanism for breaking a deadlock," says an article in *Eco*, the CAN newsletter. "No state should possess a veto over efforts to protect the global commons."

Legal experts point out that governments could choose to make decisions by majority vote, but add that this is unlikely to happen. Patrick Széll, legal adviser to the UK delegation to the climate conference, points out that, under the convention, members can call a vote "as a last resort if all efforts to reach a consensus have been exhausted".

Similarly, under the Vienna Convention on the Law of Treaties, a decision can be made by a two-thirds majority vote if a treaty's existing rules do not specify a decision-making method. But Széll points out that major states — such as the United States, China, Saudi Arabia and Venezuela — are not parties to the Vienna convention.

Governments — regardless of their views on the measures needed to stabilize greenhouse gases in the atmosphere — have held back from calling a vote on any specific issue. Mohammad Al-Sabban, Saudi Arabia's delegate to the climate conference, says that majority voting may alienate minority countries, which could weaken a treaty.

Tuiloma Neroni Slade, Samoa's representative to the United Nations and a member of the AoSIS delegation, agrees. "I'd rather not put [decisions] to the vote," he says. "Climate change is a global issue. We must solve it together."

Indeed, many developed countries, which bear most of the costs of the climate convention, appear to be opposed in private to majority voting. "The donor countries want to guard against the theoretical possibility of losing an important vote to a coalition of the smaller and developing countries," says one government lawyer.

Ehsan Masood

Japan cools conference offer for 1997

London. The location of next year's third annual meeting of the climate convention has been thrown into unexpected doubt by an announcement by Japan's delegation last week in Geneva (see above) that it was having second thoughts about hosting the meeting.

Last year, Japan announced an official "expression of interest" in hosting the 1997 conference, a move that was regarded by many as virtually a clear offer. It was seen as a potentially significant move for the meeting to take place in a country where environmental issues have not always been a high priority.

A short-list of seven cities — includ-

ing Tokyo, Yokohama, Kyoto and Kobe — had been drawn up, and a final choice was believed to be imminent. Last week, however, Japanese officials announced that no decision had been reached on hosting the conference in Japan, although they promised that such a decision would be made in two months.

"Japan needs time to discuss this issue," says Kenji Kamigawara, a member of the delegation and first secretary to Japan's mission in Geneva. Kamigawara says that any such decision will need the approval of Japan's cabinet. "We also need to ask the finance ministry to release funds."

E. M.

Politicians edge towards approval of funding for US agencies

Washington. The National Science Foundation (NSF) moved one step closer last week to permanent funding for the current fiscal year when the House of Representatives approved a bill to fund the dozens of US government departments, agencies and programmes that have been limping along on temporary spending measures. They are the casualties of the budget battle between President Bill Clinton, a Democrat, and the Republican Congress.

The bill provides funding for the rest of the year after current funding expires tomorrow (15 March). The NSF is provided under the House bill with \$3.18 billion — \$180 million less than its 1995 appropriation of \$3.36 billion in 1995. The bill also provides an additional \$40 million for basic research grants if an equivalent amount is cut from other government programmes.

The Senate is expected to vote on a companion measure this week, funding the NSF at the same level. Negotiators from the two houses will then hammer out differences between the two bills. Clinton has threatened to veto the final package if it cuts favoured programmes. The result: "We're still holding our breath," says David Stonner, an NSF legislative policy analyst. □

Japan's telecoms 'should be split'

Tokyo. Nippon Telegraph and Telephone (NTT) should be split into one long-distance carrier and two local telephone companies, according to a report released by a committee established by the Japanese Ministry of Posts and Telecommunications. The report says that adoption of its recommendations will spur competition and increase national capabilities in research and development (R&D). But critics contend that breaking up NTT — which operates 13 laboratories and spends ¥300 billion annually on research — will inhibit telecommunications research.

The report recommends that one of the newly created local telephone companies, East NTT, should inherit most of the R&D capabilities, but that research should be subsidized by West NTT — even though these companies will be competing in each other's markets. Citing the experience of the United States, NTT maintains that potentially competing companies cannot cooperate effectively on R&D. □

South Africa set to join EU research

Munich. South African scientists may gain access to most non-nuclear research programmes of the European Commission's Fourth Framework Programme by next year. Last week, South Africa started negotiations with the commission for a broad cooperation agreement that would allow its scientists to participate in European research on a project-by-project basis, provided they pay their own way. A spokesman for the commission said that the negotiations were expected to be short and straightforward.

If successful, South Africa would become the third country outside the European Union to have signed a cooperation agreement. A similar, but narrower, agreement, with Australia, covering five research areas, took effect in July 1994. An agreement with Canada, covering nine general research areas, came into force last month. □

Foresight works for UK technology

London. Technology Foresight, the British government's initiative to bring research closer to industry and wealth creation, is already "making a difference" by promoting better communication between business, academic institutions and government, according to a progress report published by the government last week.

The government has promised £36 million (US\$54 million) to fund research in priority areas outlined by the 16 Technology Foresight panels of scientists and businessmen. In addition, a number of collaborative research proposals between industry and academic institutions have been shortlisted for Foresight Challenge, a



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Improving Austrian science

SIR — As an Austrian scientist who has himself received part of his formal training in Austria and then moved on to do graduate and postgraduate work at the Massachusetts Institute of Technology in the United States, I have followed the debate on the quality of Austrian biochemical science with great interest (*Nature* 377, 468; 1995). But I disagree with the response from Hermann Esterbauer *et al.*, because they do not address the cause of the current situation (*Nature* 379, 294; 1996).

Despite the assertion that “a fair and objective evaluation of Austria’s scientific productivity and quality in comparison to its European neighbours is currently impossible”, it seems to me that the beneficiaries of the current academic system just do not like being reminded of their shortcomings. A system where external faculty appointments are the exception rather than the rule has produced an environment in which dutiful service to senior faculty, internal politics and ‘connections’ are the hardest currency in the appointments process. The mediocre candidate who has served his way through the ranks is preferred to an ambitious external candidate with a good publication record. Positions are often advertised with such specific requirements that nobody except the ‘desired’ candidate will meet the demanded criteria.

The welcome move of the Fond zur Förderung der Wissenschaftlichen Forschung (FWF) to make generous grants to young investigators provides only a superficial solution to the “misguided” appointment process. In fact, as the FWF does not provide laboratory space, young researchers are often forced to trade such space for co-authorship with more senior faculty members. This approach encourages a system of career-long dependency and servitude in which one’s professional future is linked to that of a senior faculty member. Progress has to come from providing autonomy and seed funding for startup laboratories able to extend their work after proven productivity, judged by an international peer review process. Young researchers should be given the opportunity to start independent groups that survive or fail on the basis of proven performance and not on how well they serve the purpose of established faculty. Resources will be diluted (as they are now) but the brightest and most talented investigators will succeed by attracting grant money and students once a truly free market of research ideas has been established.

Appointments for tenure-track positions should be reserved for researchers from other institutions and awarded on the basis of outstanding achievements. This should

prevent the blatant abuse of access privileges to equipment, students and bench space and curtail the destructive intellectual inbreeding and favouritism that I consider to be the root of the problem.

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Life on Mars?

SIR — Having proposed that the first living organisms lived on the thermal cycling inherent to convecting hot springs, and that Martian organisms might still do so¹, I strongly disagree with Newsom². From the existence of a plausible present-day energy source it follows that “the chances of finding evidence for past life on Mars” are certainly *not* “extremely remote”, as there is even a chance of finding life on Mars at present, albeit in a primitive form.

Irrespective of my model being right or wrong, the absence of a generally accepted standard model for the origin of life makes searches for extraterrestrial life both difficult — where and what to look for, how to recognize it? — and dangerous. Therefore, “the contamination of the Earth with living martian organisms is undoubtedly” *not* “unlikely”. On Earth, invading species from far-away habitats have caused and still cause numerous problems. The probable lack of a good understanding of any martian organism may make its containment very difficult.

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ISI miscount?

SIR — I am astonished that the Institute for Scientific Information (ISI) has identified a decline in internationally co-authored papers (*Nature* 379, 287; 1996). No explanation is given, however, for this observation.

It may very well be that the percentage of internationally co-authored papers has stabilized or even decreased. But such a trend may be an effect of database coverage, for example as a consequence of adding less-international journals.

Another explanation may have to do with the way in which the measure was constructed. ISI has counted the percentage of papers with more than one country listed

among the addresses. But even if that percentage is declining, the number of countries per paper might still grow. I tested this idea by logging on to the *Science Citation Index* database via the Dialog system. First, I summed the number of papers for each of 22 OECD countries. This sum of so-called ‘whole counts’ will always, as a consequence of international co-authorships, exceed the actual number of papers. Then I retrieved the number of papers for the 22-country set by using the boolean operator “or”, which excludes double counting. This number is in fact the same as summing fractional counts — each article is counted only once. In 1980, the ratio of whole counts (which numbered 442,828) to fractional counts (426,009) was 1.039. By 1994, the ratio had grown steadily to 1.111 (710,575/639,700). This means that international co-authorship is actually increasing and not declining.

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Little difference

SIR — In a recent article, Graeber *et al.* (*Nature* 379, 88; 1996) suggested that hypoxia selects for cells deficient in functional p53 tumour suppressor which no longer undergo hypoxia-induced apoptosis. Since the authors demonstrated that cell viability was almost unchanged at O₂ concentrations as low as 0.2% and only markedly decreased in a p53-dependent manner at O₂ concentrations around 0.02% O₂, we propose that anoxia (the lack of O₂) rather than hypoxia (a reduction in O₂ concentration) induces programmed cell death.

Although this might look like pure semantics, distinguishing between hypoxia and anoxia has important biological consequences: while some genes such as erythropoietin or vascular endothelial growth factor are already induced by physiological hypoxia (caused by, for example, high altitude or during development), others such as p53 are induced only by pathological anoxia (caused by, for example, ischaemia or extensive tumour growth) leading to cell death. As ‘real’ anoxia (0% O₂) is seldom achieved in biological systems, distinguishing between hypoxia and anoxia is functional rather than physical and the borders may overlap. It is comforting to know, however, that we do not turn apoptotic when we go skiing in the Swiss Alps.

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Errors of judgement at Greenwich in 1796

J. D. Mollon and A. J. Perkins

The origins of experimental psychology can be traced back to 1796, when the then Astronomer Royal dismissed his assistant for making some seemingly inaccurate measurements. But there is more to the story than meets the eye.

THIS year marks a bicentenary significant for both astronomy and cognitive science. In the winter of 1796, the 63-year-old Astronomer Royal, Nevil Maskelyne, dismissed his 24-year-old assistant, David Kinnebrook, on the grounds that Kinnebrook differed from him by 800 milliseconds in judging stellar transits — that is, in estimating the moment a given star passed the meridian wire in the Greenwich telescope. The incident, recorded in the printed version of the Greenwich observations¹ and noted by von Lindeneau in 1816 (ref. 2), prompted Bessel at Königsberg to study differences between himself and other well-practised observers³. Bessel introduced to astronomy the concept of the 'personal equation', an attempt to correct for the constant errors of particular observers, and his measurements led to the general realization that perceptual and cognitive processes took a quantifiable time. This astronomical interest in the personal equation in turn gave rise to the studies of reaction times and order judgements that dominated the first laboratory of experimental psychology, founded by Wundt in Leipzig in 1879 (refs 4–6); and chronographic instruments, developed by astronomers to minimize personal differences, provided the necessary apparatus^{7,8}. Historians have taken Kinnebrook's dismissal to be the event that gave birth to experimental psychology^{9,10}. Drawing on previously unknown correspondence and a new analysis of the raw data, we here re-examine the events around 1796.

Transit observations at Greenwich in 1796 were made with a telescope of eight-foot focal length constructed by John Bird of London, installed in 1750 and mounted between masonry piers with the optical axis in the north–south meridian. In the image plane of the telescope were mounted five vertical wires, the central wire corresponding to the meridian. Judgements were made by the well-tried 'eye-and-ear' method of Maskelyne's predecessor, James Bradley^{1,11}. As the star (or other object) approached each wire, the observer noted the position of the second-hand of the transit clock (which had a one-second beat). He then began counting the beats, and noted the distance of the star from the wire on the beat before the transit and its distance from the wire on the beat after the transit. He

then mentally translated the ratio of the two spatial intervals into a temporal ratio, so estimating the moment of transit. He then prepared for the next wire, adjusting laterally the ocular of the telescope so that it was optically centred on the wire currently being used. The right ascension of the star was estimated by reducing the five separate readings to give an average time for the passage of the central meridian wire. The interval between the readings

method of observing, but rather suppose that he fell into some irregular and confused method of his own, as I do not see how he could have otherwise committed such gross errors.

Kinnebrook returned to Norwich, and documents in the Royal Greenwich Observatory Archives reveal that Maskelyne employed him in 1801–02 as a computer for the *Nautical Almanac*, the calculations being done at home as piece work. He died a bachelor in Norwich in May 1802, still only 30 years old^{12,13}.

In the literature of experimental psychology, the discrepancy between the estimates of Maskelyne and Kinnebrook is often attributed to 'prior entry', a phenomenon of selective attention: an event arriving on a channel to which we are attending is perceived as earlier than a concurrent event arriving on a channel to which we are not attending¹⁴. Modern experiments confirm the existence of prior entry for discrete events, but the subjective displacements are of the order of 50–100 msec (ref. 15). An alternative view, traceable to Bessel himself³, is that time is lost in the switching of attention from one channel to the other: the observer who attends primarily to the clock will switch his attention at the critical beat and will find the

star at a more advanced position than it was at the true instant of the beat. A switch of attention may take around 300 msec (ref. 16). So two observers switching in different directions could partly account for the 800-msec discrepancy between the estimates of Maskelyne and Kinnebrook.

Kinnebrook's dismissal is given a rather different complexion by extant letters he wrote to his schoolmaster father in Norwich and to other relatives, copies of which were secured in 1985 by the Royal Greenwich Observatory. From the start, Kinnebrook's social relationship with Maskelyne was awkward. Soon after Kinnebrook arrived, in May 1794, Maskelyne raised the issue of whether the assistant should dine on his own or with Maskelyne and his family: "I might choose which I pleased", Kinnebrook tells his father, "but finding from the drift of his discourse that it was his wish that I should dine by myself I therefore told him that I could do that which he thought most convenient"¹⁷.

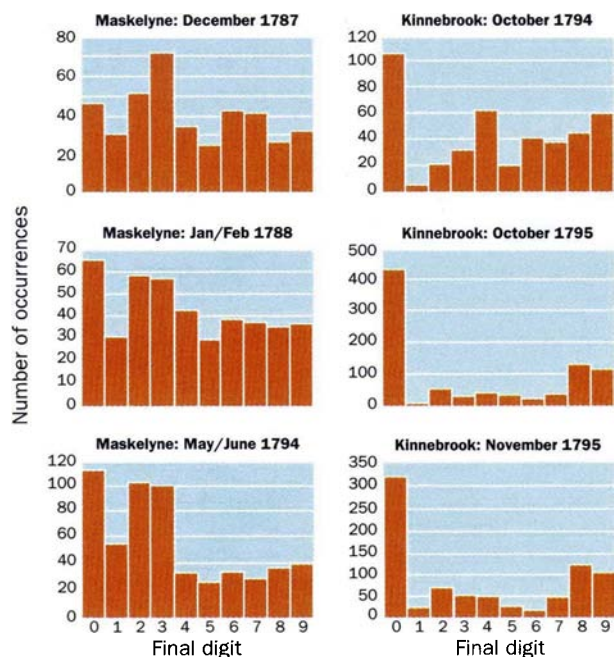
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Fifth Astronomer Royal: Nevil Maskelyne (1732–1811).

depended on the declination of the star but (in the data we analysed) had a mean of 39.5 seconds. We estimate that the spatial interval travelled between two clock beats was always less than 20 minutes of visual angle (so spatial error is possible).

Maskelyne believed that the right ascension could be estimated with a precision of the order of 100 msec. Kinnebrook's 'error' of 800 msec was serious. For on the transit judgements depended the running of the Greenwich clock. On the clock depended estimates of longitude. And on longitude depended the British Empire. Maskelyne wrote¹:

As he had unfortunately continued a considerable time in this error before I noticed it, and did not seem to me likely ever to get over it and return to a right method of observing, therefore, though with reluctance, as he was a diligent and useful assistant to me in other respects, I parted with him.... I cannot persuade myself that my late assistant continued in the use of this [Bradley's] excellent



Histograms showing for Maskelyne (left) and Kinnebrook (right) the number of readings in which a particular final digit was used. Both men have marked biases, but in the autumn of 1795 Kinnebrook is rounding many of his observations.

We learn that in October 1794, Kinnebrook broke the fourth perpendicular wire of the transit instrument "either by putting in the compound Eye Glass too far into the tube of the Telescope, or by a slight touch with my Finger"¹⁸; and in January 1795, during very cold weather, he broke the plumb line of the south mural quadrant¹⁹. Both events attracted rebukes from Maskelyne, who also disapproved of Kinnebrook's recreational activity, the submission of solutions to mathematical puzzles in the *Ladies' Journal*²⁰. When Kinnebrook asked for time off in August 1795, Maskelyne "said it would be very disagreeable to him as the Moon would not transit the mer. till near midnight on Friday and Saturday next and as it is necessary there should be stars observed both before and after the moon's transit, he would be obliged to stay up till between 1 and 2 in the morning"²¹. And in November 1795,

while Maskelyne was on his annual visit to Wiltshire (where he enjoyed the income of a living), Kinnebrook entered into a correspondence with Herschel about a new comet observed by the latter. "Dr Maskelyne was much displeased and hinted as if I had kept up a regular correspondence with Dr Herschel. If I had known there had subsisted jealousy between Dr Maskelyne and Dr Herschel I certainly should not have written to Dr Herschel about the Comet"²².

It is in the same letter, of 17 December 1795, that we first hear of Mrs Wilkinson. "Mrs Wilkinson Dr Shepherd's niece has been at the ob about 4 months. She dined with us when my Uncle was at the ob. I apprehend there is a

scheme planned between Dr M and Dr S to marry Mrs Wilkinson to an assistant." There was but one assistant at the observatory. As pressure was put on him to marry Maskelyne's protégée, Kinnebrook sought his father's advice: "I think it would be best for you to write me upon a thick sheet of paper or else send me a double letter". In a letter of 10 January 1796, Kinnebrook records: "Dr M recommended Mrs... to me as a very prudent woman and urged me very much to have an interview with her on the Tuesday morning". Kinnebrook replied that his father recommended him to continue single²³. Within a fortnight he was dismissed for having fallen into "a vitious way of observing the times of the Transits too late"¹.

Personal relationships apart, what do the raw data of the manuscript transit book²⁴ reveal about Kinnebrook's dismissal? Although we do not have an exter-

nal measure of the absolute accuracy of the observations by Maskelyne and Kinnebrook, clear conclusions can be drawn from the internal structure of the data.

Take the distributions of the final digits used by the two observers. It is known in other contexts (for example, in sphygmomanometry) that operators do not use the digits 0-9 equally when reading dials or scales to a tenth of a division: a given operator will show an unconscious preference for certain digits and this signature may be reasonably stable over time^{25,26}. The figure shows for Maskelyne the frequency of use of different digits in two successive periods in 1787-88. (The final digit corresponds to tenths of a second.) The distributions are significantly non-uniform ($\chi^2 = 45.16$; 31.54 ; $p < 0.001$). The departure from uniformity is not gross, but he has a preference for 0, 2 and 3. By May 1794, just before Kinnebrook starts work, Maskelyne has become more biased ($\chi^2 = 177.8$; $p < 0.001$); he now has a stronger preference for 0, 2 and 3 and is avoiding the larger numbers. A sample of data from Kinnebrook in the autumn of the same year shows a bias ($\chi^2 = 164.7$; $p < 0.001$) different from, and more extreme than, Maskelyne's: he has a strong tendency to round to the nearest second and almost never uses the digit 1. A year later, just before dismissal, he is rounding most entries and seldom gives an estimate to the nearest tenth.

Another way of analysing the data is to consider the standard deviation of the four intervals between the five successive transits used to estimate each right ascension. In May 1794, when Maskelyne was between assistants, the median standard deviation of his readings was 269 msec. The corresponding value for Kinnebrook in his first autumn (October 1794) is 353 msec, and by October 1795 it was 461 msec.

Neither prior entry nor attention switching can fully account for Kinnebrook's error: his judgements are not simply displaced in time relative to those of Maskelyne. Rather, it is as if his perception of the beat migrates in his perceptual memory to coincide with the moment of occultation of the star by the wire. This phenomenon recalls the tendency of a click presented during a spoken sentence to migrate so as to coincide with the break between two clauses²⁷. Certainly, Kinnebrook was not a distinguished observer. The pretext for his dismissal was sound, although the real reason for his dismissal may not be the one historically assumed. □

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ACKNOWLEDGEMENTS. We thank D. Dewhirst, D. Jones and L. Morrison for advice.

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Daisy gives an evolutionary answer

Jared M. Diamond

It is not often that evolution by natural selection is actually witnessed in plants or animals. A study of little weeds on Canadian islands now provides a striking example of the phenomenon.

CREATIONISTS attack evolutionary biologists for only inferring evolutionary change without observing it directly. Biologists respond that the large-scale evolutionary changes of most interest to us simply cannot be followed, because they take place over times far longer than a human lifespan. In a remarkable paper (*Journal of Ecology* 84, 53–62; 1996), Martin Cody and Jacob Overton now report the direct detection of both rapid evolutionary change and the 'founder effect' in populations of wild plants. (The founder effect is the phenomenon whereby new colonies of a species may become instantly distinct from the parent population, as a result of their few founding individuals being an atypical sample.)

The evolutionary phenomenon they studied is the loss of dispersal ability by plants and animals confined to islands. Many species that are endemic to remote oceanic islands lack any visible means for having reached the island in the first place (S. Carlquist, *Island Biology*, Columbia University Press, New York; 1974). These species include large flightless birds such as the now-extinct dodo and many surviving rails, as well as trees with gigantic fruits that are intolerant of saltwater and are far too big to be blown by the wind or carried by a bird.

Many biogeographers assume that these island populations were founded by colonists from mainland populations that were well adapted for dispersal, such as small flying birds or weedy plants with small wind-dispersed seeds. Once an island population is established, several selective factors would then drive a reduction in dispersal ability. For example, sedentary individuals would remain on the island and contribute to future generations; highly mobile individuals would tend to end up in the ocean and leave no progeny; and stable insular environments would select for high competitive ability at the expense of dispersal. However, with-

out direct demonstration of losses of dispersal ability with time, other biogeographers are sceptical of this reconstruction, instead suggesting that already flightless ancestors arrived from the mainland over land bridges, then both the mainland ancestors and the land bridges vanished. So many land bridges have been thus postulated that the oceans would have been criss-crossed with land if all of them had existed.

Cody and Overton sampled some 240 islands off the Pacific coast of Canada, ranging in area from a few square metres up to 1 km². A flora of short-lived

relation by measuring the ratio of pappus volume to achene volume (V_p/V_a , which reaches values as high as 17,000) for 20 diaspores from each of 155 plant populations, dropping each diaspore in still air from a height of 2 m and measuring the drop time (T_d) before it hit the ground. In

a vacuum, or for a cannonball in air, T_d for that height would be a mere 0.6 seconds. But for the fluffy light diaspores, T_d is much larger and is correlated with individual variation in diaspore morphology within each species. For instance, T_d of the weed *Hypochaeris radicata* increases from 3 to 6 seconds as the ratio V_p/V_a increases from about 1,000 to 17,000.

That doubling of drop time would presumably permit the diaspores to be carried twice as far by the wind.

These humble Canadian island weeds show the reduced dispersal ability that one normally associates with endemic species of distant oceanic islands such as Hawaii. The weeds *H. radicata* and *Lactuca muralis* both have smaller parachutes (V_p respectively 34% and 28% less) on the islands than on the mainland, while *H. radicata* also loads a heavy achene on the parachute (V_a 25% greater). What intrigued Cody and Overton about

these evolutionary changes was the possibility that they had developed very quickly, because many of the populations consisted of just a few dozen individuals and were likely to be short-lived.

This possibility was confirmed by taking a census of island plants for eight summers over the course of a decade. Population turnover proved high, with populations often becoming extinct and new ones becoming established. The new populations evidently represented fresh colonizations rather than just reappearances of old populations from buried seeds, because the initial populations invariably consisted of just one or a few individuals and appeared after absence of



A Ross islet, one of the 240 or so islands involved in Cody and Overton's research, and (inset) *Lactuca muralis*, one of the species studied.

weedy plants lives in disturbed habitats on the adjacent mainland, including many species of the daisy family (Asteraceae) which have wind-dispersed seeds. The dispersal unit of these daisies, the diaspore, has two parts: a tiny seed within a covering (the achene), surrounded by or connected to an enormously larger ball of fluff (the pappus). The diaspore thus constitutes a parachute that is well adapted to being carried by the wind, as is familiar to anyone who has watched wind-borne dandelion fluff.

The bigger the fluff ball and the lighter the seed, the longer the diaspore remains aloft, and the farther it is likely to be carried. Cody and Overton quantified this

Photos by Martin Cody

the species for up to 9 years. Cody and Overton were thereby able to measure parachute and payload sizes in populations of known ages, from an age of 1 year (just founded) up to 10 years or more.

For *L. muralis*, the species with the largest sample size, the youngest island populations prove to have significantly smaller achenes (by about 15%) than do mainland populations. This represents a founder effect: among mainland individuals, those with smaller achenes (lighter payloads on their parachutes) are more likely to get blown to islands. Achene size then increases with population age, until by about 8 years insular achenes return to mainland sizes. Conversely, parachutes (V_p) decrease in size with population age and become significantly smaller than mainland values by about 6 years. Because V_p decreases and V_a rises with population age, the ratio V_p/V_a decreases and so do drop times — probably at least in part because plant individuals with larger parachutes, lighter achenes and longer drop-times tend to remain aloft for longer, get blown out to sea, and fail to contribute to the next generation. These weeds are mostly biennials, so that all of these evolu-

tionary changes are taking place over just 1–5 generations.

Although weeds lack the exotic appeal of Galápagos finches, they constitute much more tractable systems for studying evolutionary change. They are not endangered, protected or confined to remote locations; and they can be ground up for DNA analyses, and experimentally exterminated or introduced into experimental plots without legal or ethical obstacles. Similar advantages apply in principle to a wide variety of other ephemeral small populations of small plants and invertebrates. It is ironic that, after sweating to gain insights from endemics of remote islands for a century and a half, island biogeographers are only now appreciating the virtues of backyard weeds as study objects.

Meantime, Cody and Overton's study is likely to prove highly influential. It is an especially clear and simple example of evolution through natural selection, witnessed historically. □

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MATERIALS SCIENCE

Harder than diamond?

Robert W. Cahn

THE design of exceptional materials from first principles is the philosopher's stone of the modern materials scientist, but alas for ambition, improvements in useful materials have not often sprung from theoretical inspiration. Nevertheless, one very active new experimental research field does spring directly from a theoretical precursor: the search for a superhard carbon nitride. Indeed, it is more complicated than that — theory has spawned experiment, and the recent disappointments of the experimentalists have stimulated the theorists to renewed efforts, as exemplified by a paper by Teter and Hemley on low-compressibility carbon nitrides¹.

The search for carbon nitride sprang

from a much-cited trio of papers by Cohen and Liu^{2–4}. They made calculations from first principles, on the basis of the shortness and predominantly covalent character of the C–N bond, to predict a very high elastic modulus and correspondingly high hardness for a postulated compound, C_3N_4 , with a structure equivalent to Si_3N_4 . The correlation between modulus and hardness in ceramics has generally proved to be close.

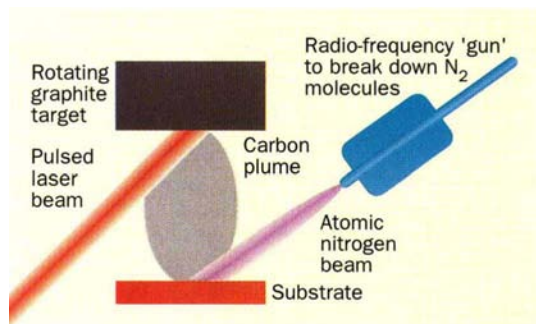
A comparison of bond characteristics in the two compounds indicated clearly that the carbon nitride should be much the harder — indeed, harder than diamond, and considerably harder than the next best candidate in the hardness stakes, cubic boron nitride. Marvin Cohen's papers, with Liu, occasioned great excitement among many physicists, chemists and materials scientists, and a large number of studies, both experimental and theoretical, have since been devoted to the carbon nitrides (C_3N_4 is only one of the currently predicted compounds, and even a fullerene-like version has been proposed⁵). After six years' work, the very existence of any carbon nitride is still disputed, but the search for this family of

compounds continues at an accelerating pace.

Teter and Hemley examined models of carbon nitride in silicon-nitride-like configurations (two subtly different hexagonal versions, α and γ), as well as a graphitic version and a cubic version based on the structure of high-pressure willemite, Zn_2SiO_4 . From a quantum mechanical treatment of the different possible electron density functions, they calculated the elastic constants of this postulated cubic C_3N_4 and found it to meet the Born criterion, which checks for stability against loss of rigidity and collapse into another crystal structure or an amorphous configuration. Their value for the bulk modulus is 496 GPa, compared with experimental and theoretical values for diamond of 442 and 468 GPa respectively. All that remains is to make the substance. Teter and Hemley calculated the effect of pressure on the relative stability of the various postulated forms of C_3N_4 , and predicted that the cubic form would be stable at high pressure. They conclude that "high-pressure synthesis should be important in the search for new carbon nitrides", and they opine that such phases might be pressure-quenchable, that is, they might remain stable if brought down to low pressures quickly enough.

I turn now to the experimental attempts to prepare carbon nitrides. Fang has written a survey⁶ on the " β - C_3N_4 search", including studies made long before Liu and Cohen's papers; he noted the great difficulty of inserting a sufficient proportion of nitrogen into the material and concluded that "there is no credible evidence of this incredible material with chemical composition C_3N_4 and an isostructure of β - Si_3N_4 ". A few months later, in the same journal, a Chinese group⁷ claimed success in making just this compound by graphite ablation in an atmosphere of atomic nitrogen (see below), using infrared spectrometry and X-ray diffraction to characterize the composition and structure of their polycrystalline films. In Australia another group⁸ claimed success in making a film with equal amounts of C and N — the highest nitrogen ratio yet achieved — using a reactive sputtering approach (C and N atoms are vaporized from solid targets by ion bombardment into the same space, allowing them to react). However, the structure is graphite-like (having large, flat monolayers weakly bound to each other) and thus not likely to be hard. Similar structures have been made by nitrogen implantation into diamond-like carbon films⁹.

The most persistent attempts to form carbon nitrides, by a variety of approaches, have been made by the American chemist Charles Lieber and his group¹⁰; very recently he has published a detailed survey of the current state of his own and other people's experiments¹¹. The principal methods have been reactive sputtering and laser-ablation



Synthesis of carbon nitrides by laser ablation. Inside a vacuum chamber, carbon and nitrogen are allowed to react at the substrate surface. (Adapted from ref. 10.)

of carbon from a graphite surface in the presence of atomic nitrogen. The ablation apparatus is shown in the figure.

Only the second approach seems to be useful for going beyond the C_2N composition. Using the ablation method, deposits close to C_3N_4 have been made, but characterization (especially by electron energy loss spectrometry) indicates that such films have a large proportion of carbon triple-bonds, which weaken the material. Only films with very low nitrogen concentrations are found to be "extended covalent materials without [triple bonded] impurity phases".

The most novel feature of Lieber and Zhang's experiments, however, is that the proportion of such triple bonds can be sharply reduced by annealing. In particular, films with the C_2N composition can be entirely cleared of triple-bonded impurity phases by this method. Studies of such annealed films have shown that diamond-like electrical properties can be achieved, but the few hardness measurements made on carbon nitride films produced by sputtering or laser ablation have shown disappointingly low values: the hardness actually decreases with increasing nitrogen content. However, composite films containing both carbon nitride and titanium nitride (a familiar phase) can have very high hardnesses¹². TiN was initially introduced here as a promising substrate to encourage formation of C_3N_4 by providing a structural template, but the formation of an intimate composite was found preferable. Hardness values of 45–55 GPa were achieved — better than cubic boron nitride and close to the bottom of the range of hardness of thin diamond films.

The conclusion is that, up to now, Liu and Cohen's proposed superhard phase has proved a will-o'-the-wisp, but neither the theorists nor the experimentalists have shown any signs of giving up the hunt. The story has demonstrated that predicting the properties of an unknown phase is one thing, but making it is quite another. □

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Now blows the east wind

Chung-I Wu

DURING the Three Kingdom period towards the end of the mighty Han dynasty, the three forces were ready for their Armageddon at the Vermilion Cliff in eastern China. Through shrewd manoeuvres, the Southern Alliance was able to dupe its enemy on the west flank into locking all of its warships into a tight formation. What was then missing on this tranquil water was a wind from the east, to send down fireships to set the whole enemy fleet ablaze.

Since then, "all ready but the east wind" has become a metaphor for action for which all preparations humanly possible have been made, but which requires nature to yield just one more thing. The discovery by Davis and co-workers of fertile hybrids between the two best-studied species of *Drosophila*, described on page 157 of this issue¹, may be the east wind in the genetic analysis of speciation.

How one species splits into two is one of the central questions in evolutionary biology. Among all differences between species, the traits contributing to reproductive isolation are the most intriguing. Although in itself the phenomenon seems to make no sense (what good does it do to produce sterile progeny?), reproductive isolation is crucial for nascent species to continue their divergence without their unique innovations being lost by blending through gene migration. What sort of genetic changes underlie this process?

Studying the genetics of reproductive isolation may seem to be an exercise in self-defeat; after all, we are attempting to study the sterility of crosses between species by means of crossing. Cases of unisexual sterility, generally obeying what is known as Haldane's rule, do however allow geneticists to practise their trade. For example, the yak–cow cross results in sterile males and fertile females. So do crosses between guinea pigs and cavies.

A more serious problem is that the traits of reproductive isolation are genetically complex, as has been known since the 1930s (refs 2, 3) and confirmed recently⁴. What this means is that even the best genetic tools available in any species may only be barely adequate. Unfortunately, *Drosophila melanogaster*,

the species for which we have one of the best genetic toolkits, cannot be used in research on reproductive isolation because it produces sterile hybrids with all known relatives, including the closest one, *D. simulans*.

This is a topic with a long history. As Davis and colleagues remark, back in 1920 A. H. Sturtevant must have been disappointed by the sterility between *D. melanogaster* and *D. simulans*. There were more disappointments to come. H. J. Muller, together with G. Pontecorvo, devised a clever way of bypassing sterility



A 'zonkey', a cross between a zebra and a donkey. Interspecific hybrids such as this are often sterile. Why? (Photograph: Derek Bromhall/Oxford Scientific Films.)

in the first filial generation (F_1) to obtain genotypes equivalent to the backcross F_2 generation. They were essentially all sterile³. In the 1970s, Tsacas and colleagues searched for more relatives of *D. melanogaster* and found a pair of sibling species from islands in the Indian Ocean, *D. mauritiana* and *D. sechellia*⁵. Both, like *D. simulans*, produce sterile hybrids in their hybridizations with *D. melanogaster* (although in other respects the three species have been useful in research on reproductive isolation^{4,6}). A third attempt also failed to overcome the sterility barrier, but it finally led to the new discovery¹. Watanabe⁷ first identified a mutation that can rescue the otherwise inviable hybrids between *D. simulans* and *D. melanogaster*. A total of four such mutations have been discovered since^{8,9}. Again, however, none of the hybrids is fertile.

The existence of viability rescue mutations did offer a ray of hope: the possibility of rescue of female fertility. It was during the pursuit of one such mutation that Davis et al. discovered one last piece that may finally bring *D. melanogaster* into

COMETS

Hale-Bopp looks like a winner

Harold A. Weaver

work on reproductive isolation. They found that females of a few *D. simulans* strains, when mated to males of a particular *D. melanogaster* line, produced F_1 hybrid females that are fertile. There have been unconfirmed rumours that this has been done before, but the report of Davis *et al.* is the first to show convincingly that the strains belong in the correct species and that the F_1 and F_2 are true hybrids.

Regardless of the genetic basis of the fertility rescue, which is very interesting in itself, the discovery of this phenomenon is promising. If chromosomal segments can be individually introduced from *D. simulans* into *D. melanogaster* through fertile F_2 offspring, rigorous genetic analysis can be carried out for all aspects of species differentiation, including inviability, sterility, sexual behaviour and so on. The availability of chromosome rearrangements, mutations and molecular tools in *D. melanogaster* will make this feasible. There are still some difficulties; for example, the range of genotypes of the fertile F_2 females is not known yet. Nevertheless, surmounting them will only take human efforts.

Finally, the observation that the best rescue strain from *D. simulans* is of African origin is intriguing. Lines of *D. melanogaster* from some parts of Africa exhibit substantial divergence in DNA variation from strains from other continents¹⁰, and together these results support the view that this species group originated in that continent⁵. Could Africa contain a hidden wealth of materials for speciation research?

Our understanding of the nature of species difference as a genetic phenomenon is still primitive⁴, in comparison with the spectacular advances that have been made in understanding development. Lack of suitable materials has been an impeding factor. But with the appearance of the paper by Davis *et al.*, there is good reason to believe that the east wind is blowing (as indeed it did at the Vermilion Cliff), and that we will see significant progress in our knowledge of reproductive isolation. □

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THE excitement continues to build as comet Hale-Bopp heads for perihelion in the spring of next year. When the comet, officially known as C/1995 O1, was discovered this past summer it was exceptionally bright considering that it was about seven AU from the Sun — still well outside the orbit of Jupiter (one AU, or astronomical unit, is the mean radius of Earth's orbit). The dust production rate was one of the largest ever observed for any comet, at any heliocentric distance¹, producing a coma at least 10 times brighter than the

comparable to that produced by Halley's comet near 0.9 AU. Since sublimation rates of cometary ices generally vary linearly with solar irradiation, which will increase by about a factor of 50 between the autumn of 1995 and the spring of 1997, there is good reason to be optimistic that Hale-Bopp will put on a fine display in the spring of 1997. It reaches perihelion 0.9 AU from the Sun on April Fool's Day, 1997.

The apparition of Hale-Bopp is a unique opportunity to investigate the sublimation behaviour of cometary nuclei. Water is thought to be the dominant ice in comets, but water outgassing should be very weak at the low temperatures (~110 K) expected for heliocentric distances of 6 or 7 AU. A search for ultraviolet emission from OH, a product of the dissociation of water by sunlight, was unsuccessful¹, but a water-ice absorption band was detected in the infrared⁴. So, although CO is driving Hale-Bopp's current activity, water sublimation will presumably take over this summer or autumn when the heliocentric distance drops below about 3 AU.

H. Weaver, P. Feldman & NASA

IMAGE
UNIMAGEABLE
FOR A COMET
FOR A COMET
REASONS

Hale-Bopp's coma, with jet and spiral arm. The nucleus is within the brightest area at the centre. Sublimation of carbon monoxide in an active area of the nucleus drives off dust, which reflects sunlight to form the coma. Its morphology is like that of a tilted water sprinkler: there is a radial jet extending ~1.4 arcsec from the nucleus and a spiral arm breaking off from the tip of the jet. This image was taken on 26 September 1995 by the Wide-Field Camera on the Hubble Space Telescope.

strongest outbursts from Schwassmann-Wachmann 1, another famous trans-jovian comet. A worldwide observing campaign began immediately after Hale-Bopp's discovery. As reported by Biver *et al.* on page 137 of this issue², and almost simultaneously by Jewitt, Senay and Matthews³, the nucleus is a prodigious source of carbon monoxide (CO), and this CO outgassing is almost certainly what is driving off the dust that produces Hale-Bopp's bright coma.

Using very sensitive millimetre-wave telescopes in Spain and Hawaii, both groups detected a CO rotational line and estimated that the comet was shedding about 1,000 kilograms of CO every second. At about 6.5 AU from the Sun, Hale-Bopp was producing CO at a rate

during ground-based optical spectroscopy of Hale-Bopp, and the production rate relative to CO was about 20 times smaller than that observed for comets near 1 AU from the Sun. That and the non-detection of the other molecules imply that the vaporization behaviour of trace cometary ices is quite different from that expected for pure ices, and that CO is less easily retained in the water-ice matrix than other species. Continued monitoring of Hale-Bopp to catch the onset of outgassing for many different molecules will be one of the most important scientific objectives during this year's observing campaign.

The jet and spiral structure in some optical images of Hale-Bopp (see figure) apparently result from the periodic

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Hyakutake: an uncertain curtain raiser

It has been two decades since the spectacular comet West graced our skies in 1976. If we're lucky, the long drought may be over. As described in the accompanying article, Hale-Bopp is very likely to be an outstanding performer in the spring of 1997. On 30 January, Japanese amateur astronomer Yuji Hyakutake discovered another comet (his second discovery in slightly over one month) which may become a naked-eye object this month.

Officially designated C/1996 B2, Hyakutake will pass within 0.1 AU (15 million km) of the Earth on 25 March. If the current brightness predictions hold it will be the most active comet to pass so close to the Earth since 1556 (B. Marsden, *IAU Circ. No. 6329*). Hyaku-

take's visibility is especially favourable for Northern Hemisphere observers from late March until mid-April. It passes within a couple of degrees of the North Star on 27 March and so will be visible all night. After perihelion on 3 May the comet becomes a Southern Hemisphere object, but it will be about 10 times farther from the Earth.

Observations on 10 February of near-ultraviolet emission from OH (D. Schleicher, *IAU Circ. No. 6311*) and millimetre-wave HCN emission (H. Matthews, M. Senay & D. Jewitt, *IAU Circ. No. 6318*) showed that Hyakutake's level of gas production was only slightly below Halley's at the same heliocentric distance. Putting Halley only 0.1 AU from the Earth would make

quite a sight! However, subsequent observations (D. Schleicher & R. Millis; M. Festou *et al. IAU Circ. No. 6333*) indicate that the comet's activity level flattened out during the next 18 days, which makes any extrapolation of its brightness into late March highly uncertain. Also, the comet's proximity to the Earth means that its brightness will be spread over many degrees of the sky, diluting its visual impact. In any event we don't have much time to think about Hyakutake as it will come and go within the next month or so. My advice is to watch for further developments (via the Internet, newspapers, astronomy magazines, radio and so on) and then go out and see the comet for yourself if it does get bright. H. A. W.

release of huge amounts of dust from the nucleus. Sekanina⁶ has modelled the coma morphology in detail and can explain its appearance as due to dust emission from a single active vent near the comet's equator. As the comet rotates the dust release rises very sharply, with a peak at local noon, and then gradually decays to a quiescent level at the evening terminator. The peak mass-loss rate in dust is at least 15 times larger than the CO mass-loss rate that drives it. Such a large dust-to-gas ratio may be a common feature of comets far from the Sun, as a similar ratio was derived⁷ for the outburst from Halley's comet at 14 AU (in contrast, the dust-to-gas mass ratio for Halley near 1 AU was between 1 and 2). So far at least three separate emission events have been detected, each lasting about 2 days and centred on 19 August, 24 September and 12 October 1995. Notice that the 18-day separation between the last two events is exactly twice the separation between the first two. Sekanina suggests that the period is 18 days but complex nuclear rotation (rotation plus wobble) means the vent is not always fully illuminated during every cycle.

If Hale-Bopp continues to brighten as expected (and observations now coming in from after the comet's solar conjunction in February, when it was unobservable, are consistent with predictions), then the perihelion period during the spring of 1997 should provide a bonanza of new

results, because of the increased sensitivities gained by observing an extremely bright object. However, we must remember that accurately predicting cometary brightnesses is notoriously difficult and uncertain. Jewitt, Senay and Matthews³ caution that the observed outgassing in Hale-Bopp could be sustained by an exposed patch of CO ice having an area of only 14 to 56 km² (the active area is typically between 1 and 10 per cent of the surface of a nucleus, and Hale-Bopp's surface area might be as large as 5,000 km²). A patch of water-ice whose area is 14 km² would probably produce a level of activity during Hale-Bopp's perihelion

passage slightly below Halley's at the same point, which would be disappointing.

On the other hand, Hale-Bopp has a period of about 4,000 years, so any 'super volatiles' in its original surface coating should have evaporated during past visits to the inner Solar System. It is unlikely to fade out during its incoming leg, as Kohoutek (a 'new' comet) did in 1974. So I figure that Hale-Bopp is a good bet to knock our socks off next year. □

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ALZHEIMER'S DISEASE

A radical vascular connection

Jonathan S. Stamler

A GREAT discovery can be defined by the breadth of its impact. That is why Furchgott and Zawadzki's discovery¹ of endothelium-derived relaxing factor (EDRF)—an activity released from endothelial cells stimulated by acetylcholine—can be classified as such. Endothelium-dependent relaxations are found in vascular beds of all organs, in arteries and veins, in large vessels and in the microvasculature. Maturation of the EDRF response is essential for normal transition from fetal to neonatal circulation. Once turned on, EDRF production is sustained throughout life with every beat of the heart, by blood-flow induced activation of the endothelium. Abnormal EDRF responses occur in many vascular diseases, and may be involved in the pathogenesis of some such as atherosclerosis and pulmonary hypertension. An abnormal EDRF response is also an

early marker of coronary heart disease.

On page 168 of this issue², Thomas and colleagues broaden the range of EDRF-related disorders. They describe a possible connection between endothelial dysfunction and β -amyloid peptide, which has been implicated in Alzheimer's disease and age-associated neurodegeneration. Thomas *et al.* find that this damage by β -amyloid can be prevented by superoxide dismutase (SOD), an enzyme whose normal function is to detoxify excessive levels of superoxide (O_2^- , an oxygen radical). Notwithstanding the known accumulation of insoluble β -amyloid aggregates in walls of cerebral blood vessels, the notion that age-related disorders of the cortical neuropile may arise from endothelial dysfunction is potentially highly important. However, it is clear that much more work must be carried out to verify the generality of these findings

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and to establish their significance.

Endothelium-derived relaxing factor has not been positively identified. Rather, it has been pharmacologically defined as an activity that is inhibited by haemoglobin (Hb) and potentiated by SOD in a 'perfusion-cascade' bioassay^{3,4} — a somewhat artificial system where the endothelium is distanced markedly from the smooth muscle. Here it has properties that are virtually identical to those of nitric oxide (NO)³. The defining features of EDRF, however, are different in the organ chambers of its discoverers¹, where intimate contact between endothelium and smooth muscle is preserved by the use of an intact blood vessel. SOD, and the superoxide radical it scavenges, have less of an effect on EDRF in this system.

The behaviour of EDRF in the intact organism is different again from that in the organ chamber. In particular, EDRF activity is retained in the face of millimolar concentrations of blood-borne Hb, which can lower the steady-state level of free NO to below the working concentration (K_m) required by target enzymes such as guanylate cyclase⁵. This occurs by Hb establishing an NO concentration gradient (hence net flux) from smooth muscle to the blood vessel lumen. Accordingly, most free NO will go the 'wrong way'. Thus, a picture emerges in which the nature of EDRF is dependent on the bioassay and its conditions; the identity of the bioactive substance *in vivo* may differ from that *in vitro*; and the endogenous substance(s) *in vivo* is not subject to the diffusional constraints imposed by Hb, nor sensitive to the continuous production of O_2^- by the vessel wall.

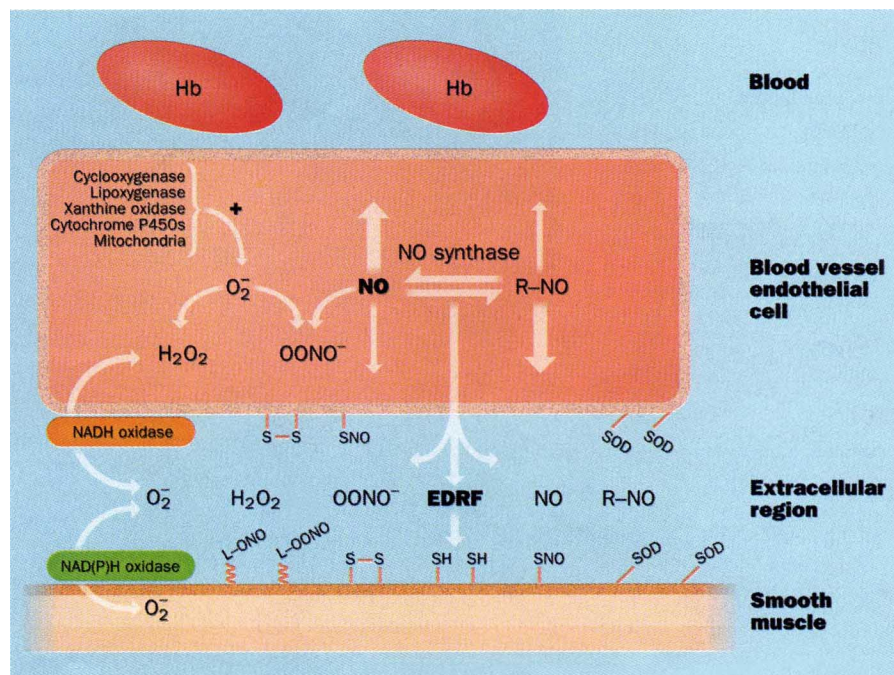
This picture may be better understood by appreciating that 'NO' activity is shared with S-nitrosothiols and metal-nitrosyl complexes⁶. These compounds maintain NO-like bioactivity in the presence of Hb and O_2^- (see figure). Their formation depends on redox state, a phenomenon that may explain the varied behaviour of EDRF in different bioassays. The notion that the nature of EDRF depends on cell redox status raises the intriguing possibility that EDRF identity varies both with vascular location and (patho)physiological condition. In this regard, it should be noted that the sensitivity of larger vessels to NO is lost in the microvasculature, whereas EDRF and S-nitrosothiol responses are maintained⁷. Pertinent to the report of Thomas *et al.*², the EDRF response in cerebral vessels has been better equated with S-nitrosothiols than with NO⁸. Interpretation is also complicated by the existence of other endothelial relaxants and by differences in the contribution of NO-related activity to the overall EDRF response in adjacent segments of vasculature. Accordingly, it would be of interest to know which segment of the aorta (thoracic or abdominal)

Thomas *et al.* used, and whether the vasoactivity of β -amyloid can be seen in cerebral vessels.

Metabolic processes that use O_2 are commonplace in all cells, and disruption of such processes is characteristic of degenerative diseases. Atherosclerosis, or age-related disease of the vasculature, has been associated with an oxidant stress that attenuates endothelium-dependent relaxations. The potential sources of tissue oxidants include the mitochondria, xanthine oxidase, cytochrome P-450 enzymes such as NO synthase, cyclooxygenase and

heparin-binding domain, it binds to sulphated proteoglycans on the cell surface and in the basement membrane.

Insight into the mechanism by which the β -amyloid peptide increases endothelial O_2^- production may be gleaned from the observation that exogenous SOD confers protection. This places the source of O_2^- in or at the plasma membrane, as the cell is not permeable to superoxide anions. This possibility is supported by reports that β -amyloid activates the membrane-associated neuronal NADPH oxidase, and that β -amyloid neurotoxicity is



Perspective on regulation of endothelium-derived relaxing factor (EDRF). Endothelial NO synthase generates NO-related bioactivity, EDRF, in the form of NO and R-NO (where R is either a transition metal or a thiol). Free NO can be inactivated by both O_2^- , of which there are several tissue sources, and haemoglobin (Hb). High concentrations of Hb within red blood cells establish a diffusion gradient for NO away from smooth muscle and towards the blood vessel lumen. R-NOs maintain EDRF activity by resisting O_2^- inactivation and reacting primarily within the vessel wall. Production of O_2^- by the cell-surface NAD(P)H oxidoreductase is envisaged to play a role in redox signalling. Inasmuch as NO/ O_2^- reactions (like R-NO reactions) promote S-nitrosylation and/or oxidation (giving rise to -SNO or -SS- modifications) of signalling proteins that regulate smooth-muscle relaxation, normal levels of extracellular O_2^- may be viewed as a synergist of EDRF activity. The extracellular isoform of superoxide dismutase (SOD) mitigates the excessive oxidation of cell surfaces by O_2^- and peroxynitrite. Lipids modified by nitrogen oxides (L-ONOO and L-ONO), which may well have some biological function, have been identified in the vessel wall (B. Freeman, personal communication).

lipoxygenase. But the main source of endothelial O_2^- (at least in bovine coronary artery) is the membrane-associated NADH-dependent oxidoreductase⁹. Like its counterpart, the O_2^- -generating NADPH-dependent oxidase of phagocytic cells, activation can be triggered by the binding of a specific peptide to a trimeric G-protein-coupled receptor¹⁰. The endothelial enzyme, however, is distinguished by its constitutive activity. The continuous threat of oxidant damage at the outer face of the endothelial cell is met by the special positioning of SOD in the vessel wall; most of this SOD exists as the extracellular isoform¹¹. By virtue of a

mediated by its ability to generate O_2^- /H₂O₂ (ref. 12). A scheme emerges in which G-protein-coupled membrane oxidases, activated by peptide and sensitive to pertussis toxin, are responsible for the generation of reactive oxygen species at the cell surface. Examples of peptide activators include formyl methionyl peptides in phagocytic cells, amyloidogenic peptides in neurons and angiotensin II in blood vessels. It remains to be resolved whether activation occurs through binding to tachykinin-related receptors, or by virtue of the amphiphilic character of peptides that enables membrane-associated signalling pathways to be perturbed. ▶

We are left with several caveats about the new results. First, O_2^- production is only inferred by Thomas *et al.*, not measured. Second, we are told that pretreatment with SOD is necessary to protect against the toxic effects of β -amyloid: apparently, addition of SOD after β -amyloid does not confer protection. This implies that only the initial phase of the respiratory burst, in which oxygen radicals are released, occurs at the outer face of the plasma membrane where SOD can scavenge. It also implies that 'extracellular' O_2^- has to be responsible for the sustained generation of intracellular (extracellular SOD-inaccessible) O_2^-/H_2O_2 that leads to endothelial dysfunction. This may not be too far-fetched as O_2^-/H_2O_2 can activate oxidoreductase-related signal transduction involving G proteins and phospholipase D, and O_2^- production by the vascular oxidase appears to be directed inside the cell^{6,9}.

Third, the severity of β -amyloid-mediated endothelial damage, which occurred over 30 minutes, is unusual: neuronal cell death is much slower. Perhaps the affinity of β -amyloid for glycosaminoglycans¹³ might displace extracellular SOD, leading to a situation where O_2^- production is incurred by a cell disarmed of its natural defence. Nonetheless, the overt cell damage brought about within minutes may need to be reconciled with the modest endothelial dysfunction produced by β -amyloid in bioassays. Finally, the animal model needs validation: the findings shown in the rat may not hold in humans.

The studies of Thomas *et al.* highlight a critical balance between NO and O_2^- in cells. Their constitutive production at the cell membrane probably reflects their involvement in redox signalling. A relative excess of NO over O_2^- would seem to be advantageous. This condition would facilitate regulation by S-nitrosylation of pro-

teins, including the charybdotoxin-sensitive K^+ channel (direct activation by EDRF elicits relaxation), glutamate-receptor-coupled ion channels (inhibition may limit the neuronal toxicity of β -amyloid), p21^{ras} and the NAD(P)H oxidoreductase (for inhibitory feedback control)⁶. S-nitrosylation would also generate EDRF activity which is resistant to O_2^- inactivation. As the production of O_2^- increases, oxidation reactions and related signals would become more prevalent. A relative excess of O_2^- will lead to toxicity if cellular defences are overwhelmed^{6,14}.

The possibility that disorders associated with deposition of insoluble β -amyloid aggregates reflect an NO/ O_2^- imbalance has potential therapeutic implications. Treatments might include NO supplementation with O_2^- -resistant NO donors, or the elimination of O_2^- with either NAD(P)H oxidase inhibitors or SOD

mimetics (an extracellular SOD mimetic might be best). Scavengers of $OONO^-$, the oxidizing product of NO/ O_2^- , could be beneficial. Alternatively, antioxidants such as vitamin E, which both preserves endothelial function and attenuates the neurotoxicity of β -amyloid, could be tried. It will be interesting to see whether β -amyloid-mediated damage to the endothelium contributes significantly to age-related neurodegeneration, or whether a deficit of endothelium-derived 'NO' contributes to the clinical syndrome of Alzheimer's disease, recalling that NO may also be involved in memory. □

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HUMAN GENOME PROJECT

A march of genetic maps

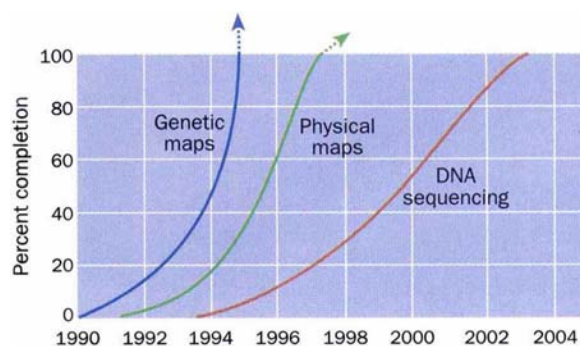
Elke Jordan and Francis S. Collins

THIS issue of *Nature* marks a further milestone in the Human Genome Project. Two papers, by a group led by Eric Lander and Bill Dietrich (page 149¹), and by Jean Weissenbach and colleagues (page 152²), describe the completion of comprehensive genetic maps of man and of the mouse, respectively. The Human Genome Project has set itself three major scientific goals (see figure): creation of genetic maps, development of physical maps, and determination of the complete sequence of human DNA. To facilitate the analysis of the human genome, the Human Genome Project has also proposed that maps and DNA sequence should be produced for a small set of model organisms (a bacterium, yeast, nematode worm, fruitfly and laboratory mouse). The publication of the two new maps effectively completes the genetic mapping phase of the project.

The human genetic map produced by Weissenbach and collaborators is distinguished from the previous comprehensive map³ by being composed entirely of markers known as microsatellites. These markers consist of short tandem repeats of two or more nucleotides, with the number of such repeats varying from individual to individual. The advantage of these markers over previous types of marker such as RFLPs (restriction fragment length polymorphisms) is that they can be assayed by

the polymerase chain reaction (PCR) under uniform conditions. The sequences of primers needed for each marker are all electronically available and it is possible to carry out large numbers of assays rapidly in an automated fashion.

To be maximally useful, genetic markers must have high heterozygosity: in other words, there should be a high likelihood that a marker is different in any two copies of a chromosome. The markers in this map have a heterozygosity of 0.7 on average, which is a very useful level. Another important characteristic of a



Progress in the Human Genome Project, showing the projected rate and time of completion for the three major goals.

genetic map is the density of coverage. The Weissenbach map contains 5,264 markers located to 2,335 positions using family data in the collection of the Centre d'Etudes du Polymorphisme Humaine (CEPH). So, on average, there is a marker every 0.7 cM (one centimorgan is equal to a 1 per cent chance that two markers will be separated during meiosis by recombina-

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nation), or about every 700,000 base pairs. This density is much better than that specified in the goal for genetic mapping set for the US human genome programme, which was achieved more than a year ago. These markers have also been incorporated into the human physical map⁴⁻⁹.

Lander, Dietrich and colleagues' mouse genetic map marks the conclusion of a five-year project. This map also consists of microsatellite markers; but because mice can be bred at will, it is based on a cross between two strains of mice rather than on natural families. A total of 6,336 markers that are different in these strains were scored and assembled into a map of 1,001 'bins' containing an average of 6.3 markers each. Having completed this map, the authors then interdigitated it with a second mouse map based on 797 RFLPs and rich in known genes. The final integrated map has 7,377 loci, on average one every 0.2 cM or every 400,000 base pairs.

Dense genetic maps make possible the identification of genes for single-gene disorders and the dissection of polygenic traits, and these two maps have already changed the face of human and mouse biology. Both groups made their markers available well before publication. So although these papers mark the completion of the maps, their impact has already permeated the genetics community.

Whether in mouse or man, mapping disease phenotypes by linkage analysis used to be a daunting task, one outside of the reach of many relatively sophisticated molecular biology laboratories. Marker sets were incomplete, the scoring required tedious and finicky Southern blotting methods, and years of work were often required to identify a linked marker. The new maps have changed all of that — mapping a single-gene disorder (typically requiring analysis of tens of thousands of genotypes) can now be accomplished in a modest-sized lab in a few months.

For polygenic conditions, the challenges are much greater, as the simultaneous tracking of several susceptibility loci, each making only a relatively modest contribution to risk of disease, requires much larger numbers of affected individuals. Such projects often need hundreds of thousands of genotypes, and can only be attempted with the kind of dense and readily scorable marker maps described here. This incipient harvest of information about genetic contributions to complex traits, much anticipated as one of the most powerful components of the new molecular medicine, is now beginning to appear. Many of the early successes, from mapping loci involved in hypertension to mapping those for certain behavioural traits, have been achieved in animal models because of the additional analytical power made possible by selective breeding schemes. Thanks to the availability of

these maps, however, promising chromosomal regions associated with polygenic human disorders such as juvenile-onset diabetes, schizophrenia and learning disability are now being identified. A rat genome project has recently been spun off, the reason being that a large number of quantitative trait models are available in that species, and a similarly dense map of microsatellite markers for the rat is expected by 1998.

What next for the Human Genome Project? The maps produced by Weissenbach, Lander and their colleagues have been compiled with care and devotion, and their value is unquestionable. Nonetheless, improvements in mapping technology may still be needed in the future. In particular, the need to run electrophoretic gels to separate microsatellite marker products by size presents obstacles to total automation of that process. Evenual replacement of these markers with an even denser map of single-nucleotide polymorphisms, which could be scored with DNA chip technology, may have significant advantages for truly high-throughput applications.

But with the genetic mapping goals essentially complete for now, we look forward to completion of the second phase, a physical map of the human genome, which is expected in about a year (see figure). This map will consist of 30,000 STS (sequence-tagged-site) markers distributed at intervals of approximately 100,000 bases along the entire genome. Physical maps of several chromosomes have been completed or are nearing completion, and late last year a preliminary physical map of the whole genome⁴ and a map with 94 per cent coverage from 15,000 markers⁹ were published.

The international genome community is now turning its attention to the ultimate challenge, sequencing the 3 billion base pairs of the human genome. This year, a number of groups will be scaling up sequencing of human DNA to a rate of many megabases a year. There is growing confidence that this most difficult of the tasks, once considered impossible by many, will also be successfully accomplished — perhaps even a bit ahead of the original target date of 2005. □

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DAEDALUS

Spin and lift

A SPINNING ball swerves in the air — a fact known and exploited in many sports. This is the Magnus effect, by which a spinning object experiences lift at right-angles to the airflow past it. Curiously enough, even fixed-wing aircraft depend on the Magnus effect for their lift. It is generated by a flow of air around each wing, back over the top surface and forward again under the lower one. Of course, this circulation is merely a small component of the much larger slipstream flowing back past the craft. It takes the form of a slightly faster backflow over the wing, and a slightly slower one under it. Daedalus is now devising an improved wing with enormous Magnus circulation.

He recalls the Flettner sailing ship of the 1920s. Instead of sails, it had big upright spinning cylinders on its deck, which generated sideways Magnus thrust from the wind blowing past them. His first idea was an equivalent Flettner aircraft, with two long horizontal spinning cylinders sticking out from the fuselage instead of wings. Sadly, it would have far too much drag. But Daedalus soon saw a way out. Imagine, he says, a conventional aircraft wing, and imagine a continuous sheet wrapped round it like a wide conveyor belt. Spin this belt rapidly round the wing, back over the top and forward again underneath, and you have an ideal Magnus-effect wing.

Not only would this cunning design have far more lift than a standard wing; its lift could be varied in flight by changing the speed of the belt. A standard wing is crowded with elaborate slots, flaps and droops, all designed to raise its lift at low speeds, so as to simplify take-off and landing. Daedalus's light, elegant 'Magnaplane' needs no such complexity. Each wing is simply a frame carrying rollers defining a fairly conventional aerofoil cross-section, and a wide belt which runs round the rollers at a speed controlled by the pilot. Daedalus calculates that at their fastest, the racing belts could generate ten times the lift of a normal wing, or (just as useful) the same lift at a tenth of the speed.

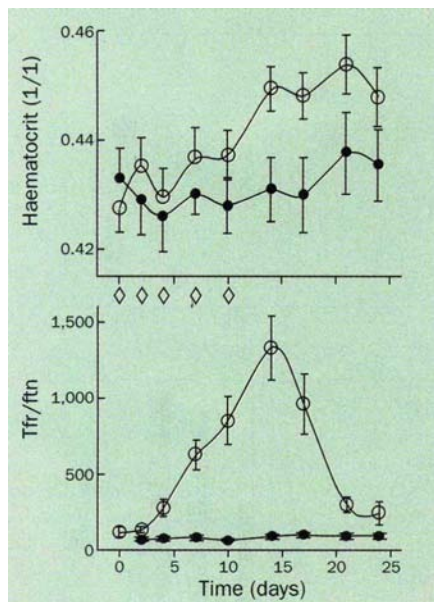
Thus the Magnaplane combines the virtues of a biplane, a helicopter and a blimp. It can land and take off from tiny fields, saunter lazily around the sky on search or surveillance missions, turn on a dime and soar like a lift. Speed up the belt on one wing, and it will bank neatly for a turn; a bit more speed and it will flip into a spectacular roll. Put both belts into reverse, and it will drop like a stone in a awesome emergency dive. Even if the belts fail, it will merely revert to a conventional aeroplane, and can fly safely home.

David Jones

Erythropoietin abuse in athletes

SIR—The availability of recombinant human erythropoietin (rHuEpo) has made it a drug of choice for athletes looking for an artificial performance enhancer. The lay press still represents the main reporting vehicle for such illicit use of erythropoietin, the most dramatic picture having been portrayed in the German weekly magazine *Der Spiegel*: 18 deaths related to erythropoietin administration among racing cyclists¹.

Although it is on the list of banned substances issued by the medical commission of the International Olympic Committee, the non-medical use of erythropoietin remains uncontrollable. No reliable analytical technique^{2,3} is available to detect its use as an ergogenic agent. The kinetic constraints (for example, short half-life, delayed erythropoietic effects) of an approach based on the electrophoretic mobility measurement of erythropoietin⁴ prompted us to investigate a new marker of erythroid activity, the soluble transferrin receptor, released predominantly from haematopoietic progenitors, in healthy athletes receiving either placebo or rHuEpo administration. Our data indicate that erythropoietin induces striking changes in the serum soluble transferrin receptor (Tfr) content. These observations could eventually be considered in the design of a probe to detect erythropoietin misuse.



Haematocrit (upper panel) and soluble transferrin receptor/ferritin⁷ (Tfr/ftn; lower panel) values in blood samples collected repeatedly (days 0, 2, 4, 7, 10, 14, 17, 21, 24) in healthy, trained, adult males (age $\pm$ s.d. 21.4 $\pm$ 0.3 yr) given 5 subcutaneous injections (○) of placebo (●; $n=10$) or 200 U kg⁻¹ commercial (Eprex, France) recombinant human erythropoietin (○; $n=19$).

Because rHuEpo administration stimulates erythropoiesis and induces the redistribution of storage iron into erythroid elements, Tfr as an index of both tissue iron deficiency and expanded erythroid progenitor mass has been expressed in relation to serum ferritin (ftn), a measure of body iron store, thus giving the serum Tfr/ftn index. This approach is particularly appealing, as the expression of such a ratio obviates problems related to the variable effects of hydration, as is the case with haematocrit readings. Moreover, changes in this Tfr/ftn index could reflect rHuEpo abuse, as well as any other manoeuvres that accelerate erythropoiesis⁵.

Analysis of variance performed on the data in the figure indicates that no significant change ($P=0.53$) in serum Tfr/ftn occurred over the entire observation period in the placebo-treated subjects, while striking increases were induced by the rHuEpo treatment. The rHuEpo-induced increases were statistically different ($P<0.05$) from basal values (pooled placebo group Tfr/ftn values) for serum Tfr/ftn values measured on days 4, 7, 10, 14, 17 and 21. This relatively low-dose rHuEpo treatment yielded no significant ($P>0.05$) increase in haematocrit values. One can speculate that the magnitude of a Tfr/ftn increase observable with a rHuEpo dose sufficient to yield an ergogenic haematocrit increase would be even more dramatic.

Notwithstanding the discriminative power of low haematocrit values observed in anaemia, patients presenting an increased serum Tfr concentration associated with primary or non-pharmacological secondary polycythaemia, or with iron-deficient or megaloblastic anaemia, are not likely to yield false-positive results, as they generally do not achieve elite-level physical performances. Physical exercise *per se* does not seem to be associated with increased Tfr/ftn serum values⁶, thus precluding a false-positive identification of erythropoietin from blood sampled at the competition site.

Hence, observation of concomitant changes in haematocrit and Tfr/ftn values could permit the discrimination of pathological from physiological conditions, and

thus distinguish between rHuEpo abusers (or even athletes who had undergone blood transfusions) and those competing fairly. The most recent technological developments already allow measurement of these discriminating variables from a few microlitres of capillary blood sampled from the fingertip or ear lobe. This first breach in athletes' immunity to detection of their use of engineered hormones as performance enhancers is a pledge in favour of the blood matrix to detect and deter sophisticated abusers.

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Derivative of the hyperbolic cotangent

SIR—The derivative of the hyperbolic cotangent is a standard result which appears in essentially every compilation of mathematical formulas¹. Here we point out that this result is incomplete; there is, in fact, an additional term which is proportional to the Dirac delta function. We present the correct formula, outline its proof and give an example of its importance in the analysis of a physical problem.

The correct formula is

$$\frac{d}{dy} \coth y = -\operatorname{csch}^2 y + 2\delta(y) \quad (1)$$

where $\delta(y)$ is the Dirac delta function. The usual derivation of the first term does not properly handle the fact that $\coth y$ has, in addition to the obvious $1/y$ singularity, a discontinuity at $y=0$. A better approach is to write

$$\coth y = \pm \left\{ 1 + \frac{2}{e^{2|y|} - 1} \right\} \quad (2)$$

where the + and - signs refer to $y > 0$ and $y < 0$, respectively. Regardless of the sign

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of y , the derivative of the second term in equation (2) gives the conventional result: the first term in equation (1). However, the first term in equation (2) can be replaced by $\theta(y) - \theta(-y)$, where $\theta(y)$ is the Heaviside function ($=1$ for $y > 0$ and 0 for $y < 0$). Since the derivative of the latter is the Dirac delta function, the second term in equation (1) follows.

As an example of the relevance of this new result in a physical problem, we refer to our analysis of a quantum particle coupled to a quantum-mechanical heat bath (which is fundamental to many fields of physics, including statistical mechanics, condensed matter and quantum optics)². We derived exact results for a very general class of models but, in the particular case of constant friction ζ , we found that the autocorrelation of the quantum-mechanical random force $F(t)$ could be written in the form

$$\frac{1}{2} \langle F(t)F(t') + F(t')F(t) \rangle = \frac{\zeta}{\pi} \int_0^\infty d\omega \hbar \omega \coth(\hbar \omega / 2kT) \cos[\omega(t-t')] = kT\zeta \frac{d}{dt} \coth[\pi kT(t-t')/\hbar] \quad (3)$$

where T is the temperature. In the classical limit ($\hbar \rightarrow 0$), use of the correct formula (1) leads to the familiar white-noise result

$$\frac{1}{2} \langle F(t)F(t') + F(t')F(t) \rangle \xrightarrow{\hbar \rightarrow 0} 2kT\zeta \delta(t-t') \quad (4)$$

However, we would get zero if we used the conventional result without the delta function; it is only by using equation (1) that we achieve agreement with equation (4).

Naturally, we were motivated to re-examine the derivatives of the other hyperbolic functions (especially the $\cosh$ since, like the $\coth$, it is also a discontinuous function), but we found that the derivative of the $\coth$ is unique in requiring modification.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words. Manuscripts can be submitted to London or Washington.

Cheaters in yucca/moth mutualism

SIR — A long-standing puzzle in the obligate pollination/seed predation mutualism between yuccas and yucca moths is that many adult female yucca moths (*Tegeticula yuccasella* (Riley)) lack functional maxillary tentacles^{1,2}, the structures used to transfer pollen. Here I report that the yucca moths without maxillary tentacles are non-pollinators, and are morphologically, behaviourally and phenologically distinct from normal yucca moths. By ovipositing in fruit rather than flowers, non-pollinators experience relatively low larval mortality, and consequently can have a great impact on seed production by yuccas.

Discriminant function analysis of characters of the female genitalia shows that adult female yucca moths without maxillary tentacles are morphologically distinct from normal yucca moths collected from the same yucca hosts (*a* in the figure; $P = 0.007$ to <0.0001), and moths lacking maxillary tentacles, but collected from different yucca hosts, are morphologically distinct ($P < 0.0001$). This provides additional support for *T. yuccasella* being a group of closely related species^{1,3}.

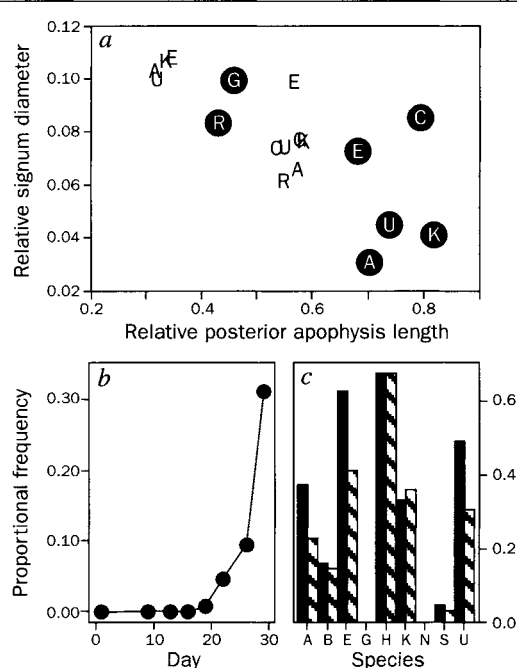
On *Yucca kanabensis* in southern Utah and on *Y. elata* in central Arizona, yucca moths without maxillary tentacles do not function as pollinators. They never attempt to collect pollen, carry pollen, approach stigmas of fresh flowers, or transfer pollen. Although they rest in fresh flowers with the pollinators during the day, at night non-

pollinators oviposit only in 10–30-day-old fruit, inserting their eggs through the carpal wall directly into developing seeds. The flight season of non-pollinators is later than that of normal yucca moths (*b* in the figure), which explains the high variation in the occurrence and relative abundance of non-pollinators from single collections of yucca moths¹.

Non-pollinators are widespread and abundant in the southwestern United States, from Texas to California. Based on collections of moths and fruit, non-pollinators occur on at least 10 species of yuccas (*a*, *c* in the figure), and in those fruit attacked by non-pollinators, their larvae were 2.13 times more abundant than pollinator larvae. Individual fruit contained up to 50 larvae of the non-pollinators, well above the 15 larvae required to damage all seeds in a fruit⁴. Considering all fruit collected, non-pollinators occurred in more than 30% of the fruit from four yuccas, and the larvae of non-pollinators constituted more than 30% of all yucca moth larvae in five species (*c* in the figure).

Two aspects of these observations deserve emphasis. First, non-pollinators are more closely related to other members of the *T. yuccasella* complex than they are to any other yucca moths or false yucca moths¹. This contrasts with the pollination/seed predation mutualism between figs and fig wasps, where pollinators and non-pollinators belong to different fam-

a, Morphology of adult female yucca moths scaled relative to wing length. Each letter represents the mean for moths of a given type and host. Symbols enclosed in solid circles represent moths that lack maxillary tentacles. From 7 yuccas with moths lacking maxillary tentacles, I selected for analysis 52 females without maxillary tentacles and 133 normal females. Letter codes for the taxa are: A, *angustissima*; C, *constricta*; E, *elata*; G, *glauca*; K, *kanabensis*; R, *reverchoni*; U, *utahensis*. *b*, Proportion of adult female yucca moths that lacked maxillary tentacles as a function of days within the flowering season for *Y. kanabensis*. Sample sizes are shown in parentheses. *c*, Proportion of fruit containing at least one larva of non-pollinators (hatched bars), and the proportion of yucca moth larvae from non-pollinators (solid bars). Data from 77 collections of fruit from 9 species of yucca. In the 2,520 fruit there were 7,388 larvae from pollinators and 3,961 larvae from non-pollinators. Grouped by species from left to right in the figure, the total number of fruit examined were 620, 536, 440, 40, 80, 520, 20, 160 and 104, respectively, and the total number of larvae examined were 1,786, 496, 2,097, 71, 228, 3,225, 16, 269 and 592, respectively. Letter codes for yucca taxa: A, *angustissima*; B, *baileyi*; E, *elata*; G, *gilbertiana*; H, *harrimaniae*; K, *kanabensis*; N, *neomexicana*; S, *schidigera*; U, *utahensis*.



ilies⁵. Second, yuccas control larval densities of pollinators more effectively than they do non-pollinators. Because about 90% of pollinated flowers abscise⁶, and because abscission of flowers can be selective⁷, pollinators experience high larval mortality. By ovipositing in young fruit, of which only about 10% abscise, non-pollinators frequently have a greater impact on seed production than do pollinators. It is of continuing interest to determine the mechanisms regulating the frequency and impact of this and other forms of 'cheating' (see ref. 8) in yucca/yucca moth mutualism.

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• See also the Letter to Nature on page 155.

Atlantic forest extinctions

SIR — High estimates of future extinction rates¹ derived from deforestation figures appear to be seriously undermined by data from South America's Atlantic forests². Here, nearly 90% of the rainforest has been cleared, yet no bird species has so far been shown to be extinct³. Is the forest-loss-to-species-loss extrapolation "simply wrong"⁴, because forest species survive in disturbed habitats⁵? Could extinctions have occurred historically, but unnoticed, as on Pacific islands⁶? Or is there a time-lag between deforestation and extinction¹, with many species now in serious danger?

The species-area relationship, $S = cA^z$, predicts how many species, S_n , of an original pool, S_o , survive a reduction in forest area from A_o to A_n ; c and z are case-specific constants. Thus, $S_n = S_o(A_n/A_o)^z$. For reasons justified elsewhere⁵, we set $z = 0.25$ and consider only endemic species. Non-endemics would survive elsewhere were the region entirely deforested^{1,5}. Using published estimates of S_o and A_n/A_o for the entire Atlantic forest^{3,6} and for endemic bird areas^{3,7} within it, we predict the number of extinctions, S_e , from $S_e = S_o - S_n$. The box above compares these predictions with the latest counts⁸ of how many endemic birds from each area are threatened by habitat loss (S_t). These threatened species were identified independently of species-area predictions, and have "a high probability of extinction in the medium-term future"⁸. We include Alagoas curassow, *Mitu mitu*, listed as "extinct in the

The endemic bird areas (EBAs) of Brazil's Atlantic forests. Data for the various EBAs are given in the table below. In the values for the whole Atlantic forest, S_o equals the sum of endemics from the EBAs ($11 + 2 + 57 + 4 = 74$) plus 140 endemics found more widely within the region. Similarly, S_t equals the sum of the threatened endemics from the EBAs ($9 + 2 + 27 + 2 = 40$), plus an additional 20 more widespread threatened endemics. The proportion S_t/S_o is not constant across these areas, but correlates with the proportion of extinctions predicted by deforestation, S_e/S_o .



Name of area	EBA code	Proportion of forest remaining (A_n/A_o)	Endemic bird species (S_o)	Extinctions predicted from forest losses (S_e)	Currently threatened species (S_t)
Alagoas Atlantic slope	B47	0.02	11	7	9
Bahian deciduous forest	B48	0.06	2	1	2
Brazilian lowlands	B51/52	0.12	57	24	27
Araucaria forest	B54	0.20	4	1	2
Whole region		0.12	214 (= 74+140)	88	60 (= 40+20)

wild", but exclude any species threatened solely by tiny ranges, direct exploitation or future (rather than current) deforestation.

Do endemic bird areas hold threatened species simply in proportion to the numbers of their endemics ($S_t \propto S_o$)? The data reject this hypothesis. In endemic bird areas with 12–20% forest remaining (B51/52 and B54), 29 endemics are threatened (32 are not), whereas in areas with 2–6% forest (B47 and B48), 11 endemics are threatened, but only 2 are not ($\chi^2 = 5.93$; 1 d.f.; $P < 0.02$). The alternative hypothesis is that the proportions of threatened species, S_t/S_o , correlate with the proportions of extinctions predicted from deforestation S_e/S_o . They do; $r = 0.72$. Consequently, for the two groups of endemic bird areas, the data do not reject the hypothesis that S_t and the non-threatened species $S_o - S_t$ equal the independently derived S_e and $S_o - S_e$, respectively ($\chi^2 = 4.01$; 2 d.f.; $P > 0.1$). Finally, as found elsewhere¹, more endemics restricted to single endemic bird areas are threatened (40 of 74) than ones found more widely within the region (20 of 140; $\chi^2 = 38$; 1 d.f.; $P < 0.01$).

If Atlantic forest endemics were adapted to survive forest fragmentation^{2,3}, we would count significantly fewer threatened birds than deforestation predicts ($S_e > S_t$). We do not. We would also count fewer threatened species if the forests lost them before they became known to science. The kinglet cotinga, *Calyptura cristata*, has not been recorded this century⁸. Other species may have escaped

documentation altogether, but our data suggest that such cryptic losses were rare.

The time-lag between deforestation and extinction is thus the best explanation for the similarity between the observed numbers of threatened Atlantic forest endemics and the numbers expected from deforestation. Forest clearance is leading to bird extinctions in these forests, at levels predicted by simple species-area analyses. Without immediate conservation action, the numerous Atlantic forest birds (and untold numbers of other taxa) currently threatened will soon be extinct.

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Splits in fruitfly *Hox* gene complexes

SIR — The homeotic genes are strikingly conserved between invertebrates and vertebrates. There is conservation, not only of the homeobox sequences, but also of their collinear order on the chromosome. The genes are found in clusters or 'complexes', and are arranged on the chromosome in the order of their function along the anteroposterior body axis (for review, see ref. 1). In the fruitfly *Drosophila melanogaster*, the homeotic genes are split into two separate clusters, the Antennapedia complex (ANT-P) and the Bithorax complex (BX-C), which direct development of the anterior and posterior segments, respectively. We show that in *Drosophila virilis*, a closely related species², the homeotic genes are also in two clusters, but the split occurs within the BX-C. The existence of two independent splits in the *Drosophila* lineage suggests that these flies lack the molecular constraint responsible for the ordered clusters in other animals.

In *D. virilis*, an *Ultrabithorax* mutation was recovered and found to be associated with a T(Y;2), which breaks chromosome 2 at map position 27F (according to the map of Gubenko and Evgen'ev³). The chromosome also carries an inversion

between positions 24E and 25G (see figure). To determine at which site the *Ubx* gene resides, we isolated two phage clones from a *D. virilis* library that carry the 5' *Ubx* exon. *In situ* hybridization to *D. virilis* chromosomes revealed that the *Ubx* region resides at 24E, a constricted region of the second chromosome (see figure). This is one of the sites broken in the T(Y;2) mentioned above.

During studies of the *Abd-B* domain of the BX-C, we recovered *D. virilis* genomic clones containing the DNA flanking the *Abd-B* homeobox. We performed *in situ* hybridization on *D. virilis* chromosomes. Instead of hybridizing to 24E, as anticipated, the phages hybridized at 26D, a more proximal site on the second chromosome, indicating that the BX-C in *D. virilis* is split (see figure).

The *abd-A* gene lies in the middle of the BX-C in *D. melanogaster*, between *Ubx* and *Abd-B*. We have cloned an *abd-A* *D. virilis* phage, and it hybridized to 26D. Thus, the *D. virilis* copy of the BX-C is split between the *Ubx* and *abd-A* transcription units.

We wondered whether the *D. virilis* homologue of the Antennapedia complex lies at 24E, at 26D, or at a third site.

We obtained two fragments of genomic DNA of the *D. virilis* ANT-P gene covering both the P1 promoter and the homeobox exon⁴. Both probes labelled the 24E constriction, at a position indistinguishable from that of the *Ubx* probe (not shown). In *D. melanogaster*, the *zen* gene lies in the middle of the Antennapedia complex⁵. Using the *D. virilis* homologue of the *zen* gene⁶ as a probe, we also find hybridization at 24E, indicating that there is a continuous cluster between *zen* and *Ubx* (not shown).

It has long been known that the homeotic genes of the ANT-C and BX-C are split in *D. melanogaster*. Furthermore, extensive genetic studies have shown that the genes of the BX-C need not be together in a continuous complex for proper function (for review see ref. 7). Our data demonstrate that in *D. virilis*, the integrity of at least part of the BX-C is not important. Mutations have been isolated in the

flour beetle, *Tribolium castaneum*, which correspond to *D. melanogaster* mutations of both the Antennapedia and Bithorax complexes. These mutations all map to a single cluster⁸, suggesting that some insects mirror the vertebrate case. The colocalization of the *Antp* and *Ubx* genes in *D. virilis* shows that these genes were together in the common ancestor of *D. virilis* and *D. melanogaster*. Similarly, *Ubx*, *abd-A* and *Abd-B* were together in the ancestor, as they are today in *D. melanogaster*. Thus, the ancestral drosophilid had a single cluster, like the current arrangement in *Tribolium*.

Perhaps the break-up in the *Hox* cluster in *Drosophila* is related to its unusual mechanism of segmentation. *Tribolium*, like other short- or intermediate-germband insects, form segments in a temporal order, from anterior to posterior⁹. In long-germband insects like *Drosophila*, the embryo is subdivided into segments all at the same time¹⁰. It has been suggested that the linear order of the ancestral *Hox* complex is mechanistically linked to the temporal order in the specification of body segments¹¹. *Drosophila* has no apparent temporal order of segmentation or of *Hox* gene expression, and thus may be freed from the major selective pressure to maintain these genes in a single complex.

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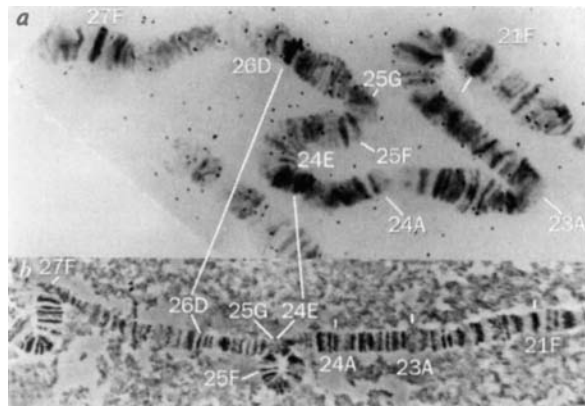
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Polytene chromosomes from salivary glands of *D. virilis*. *a*, *In situ* hybridization (bright field, $\times 93$) of the *Ubx* and *Abd-B* clones on a salivary gland chromosome from a wild-type larva. Chromosome 2 is shown. A few relevant sites are indicated. Grain clusters are visible at 26D and 24E. *b*, Squash from a larva heterozygous for the T(Y;2) chromosome associated with a *Ubx* mutation (phase contrast, $\times 23$). The same cytological sites shown in *a* are indicated. The translocation point is at 27F. The inversion between 24E and 25G carried by the T(Y;2) chromosome is visible as an inversion loop with the wild-type chromosome. Preparation of the chromosomal squashes and *in situ* hybridization using probes labelled with tritium (*Ubx* and *Abd-B*) were performed as previously described¹². Screening of phage libraries was at 55 °C in 6 $\times$ SSPE and the washes at 55 °C in 2 $\times$ SSPE. The *Ubx* probe used to screen the *D. virilis* library was the 3108 subclone spanning –31 to –34 kilobases¹³. The *Abd-B* probe was a *Pst*I–*Pvu*II restriction fragment from the cDNA pAB713 (ref. 14). This fragment covers the coding region just downstream from the CAX repeat through to the first third of the homeobox. The *abd-A* probe was a *Bam*HI–*Sal*I fragment from the phage iab-2^{c26}, which covers the genomic region from +34 to +36.5 kb¹⁵.

Chinese missile guide

David DeVorkin

Thread of the Silkworm. By Iris Chang. BasicBooks: 1995. Pp. 329. \$27.50.

AMONG the more justifiable fears of US Navy officers at the start of the Gulf War in 1991 was the likelihood that the fleet would be vulnerable to the heat-seeking Silkworm missile, which Iraq had purchased from China. The Silkworm, as it was called in the West, was part of the Haiying family of Chinese anti-ship missiles, and, along with a large portion of China's ballistic missile ordnance, was the brainchild of Tsien Hsue-shen, known (anachronistically, to be sure) in the People's Republic as "the father of Chinese rocketry". Tsien was, however, trained in the United States and had every reason to stay there if the political conditions had allowed him to. Therein lies a story.

The highly competitive and studious son of a wealthy and respected teacher, Tsien came to the United States on a Boxer Rebellion scholarship in 1935 to receive advanced training at the Massachusetts Institute of Technology. Dissatisfied with the nuts-and-bolts nature of MIT's curriculum and deeply at odds with its culture, Tsien soon moved to the California Institute of Technology in Pasadena, where he finished his studies and remained on the staff as the protégé of the theoretical aerodynamicist Theodore von Kármán, eventually rising to become the Robert Goddard Professor of Jet Propulsion in 1949. Happy in his intellectual attainments and the possibilities of life in the United States, Tsien made plans to adopt US citizenship that year.

Iris Chang follows Tsien's life from his intellectually rigorous training in China to his prominence in US aeronautics and his subsequent fate at the hands of

McCarthyism in the darkest years of recent US political history. As a result of the blind fears of that time, Tsien, after five years of confusion, humiliation and harassment, was finally deported with his family to China where he was welcomed as both hero and savant. He then did the obvious: hardened by his fate in the

public diatribes and condemnations that gave scientific credibility to Mao's absurd plans for agricultural, industrial and cultural reform.

Tsien devoted his brainpower and phenomenal discipline to the construction of a new industry, bringing to bear both management and political skills only barely mentioned in this lively account of his life. But as Chang bluntly states, she is hardly an expert in the many areas of endeavour Tsien mastered, nor is the documentary database in any way sufficient to allow yet for a definitive account. Her candour is both charming and cautionary; she never lets the reader forget the limita-

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Turning on the heat: Chinese Silkworm missile, 1987.

United States, he rose to the even greater challenge of building a competitive missile system within the political turmoil that was China under Mao Zedong.

Chang, a young writer commissioned to research Tsien's life, brings to the task talent and enthusiasm as well as fluency in Mandarin Chinese. She constantly reminds us that, without Tsien's assistance in or sanction of her project, it is not possible to establish if, how and why he changed from an idealistic theorist to a self-serving hard-line political operator willing to sacrifice everything, including valued colleagues, to gain favour with China's authorities and maintain control over its missile development. He survived and prospered during Mao's capricious anti-rightist movement, the Great Leap Forward, the Cultural Revolution and finally, in Mao's wake, the Tiananmen Square massacre, when he denounced the dissident astrophysicist Fang Lizhi as "the scum of the nation". We may never know if Tsien felt he was living a nightmare once back in China or if he was a fervent convert to Maoism. But Chang shows how he contributed to the nightmare through

tions of what is otherwise a truly engaging and poignant story.

Reflecting available documentation, including government records, oral testimony, newspaper accounts, biographical studies of Tsien and her close readings of relevant secondary sources, the author devotes most of the book to Tsien's 20 years in the United States. Nicely portrayed is his relationship with von Kármán, but more telling is how he reacted to Western cultural norms represented by the behaviour of less-capable colleagues and, eventually, by his hapless students, first at MIT, where he returned to teach briefly, and, to a lesser extent, at Caltech. Tsien is remembered by some students as a driven, enigmatic, often cruel and hurtful teacher. He could be nasty and derisive to those who failed him, including graduate students who had the audacity to take such amenities as a lunch break. His own theoretical brilliance, born of self-imposed rigour and discipline, insulated him from the world, and one can imagine his ruthlessness at the blackboard translated into political action once back in China. Just as he had no interest in practical handiwork

German rocketry

Just out in paperback is *The Rocket and the Reich: Peenemünde and the Coming of the Ballistic Missile Era* by Michael J. Neufeld. "One cannot study science and technology in the Third Reich without raising dangerous, difficult and important general questions about the relationship between knowledge and power, the moral responsibility of scientists and engineers, and the relationship between modernity and brutality.... [Neufeld] has written the first complete history in English of the story of the German liquid-fuelled rocket programme... and does not shy away from these issues", wrote David Edgerton in his review of the hardback edition in *Nature* **373**, 485 (1995). Harvard University Press, \$15.95, £9.95.

Popperfoto

as a youth, he valued theory above all else in his early career. But eventually, influenced by the small circle of rocket enthusiasts centred on Frank Malina, together with the exigencies of war in the 1940s, Tsien engaged practical issues as part of von Kármán's Scientific Advisory Group for the US Army Air Force, and evidently came to like the activity. Tsien's professional life in the United States before the McCarthy period provides a useful glimpse of international fertilization in science and engineering. One can draw parallels between his experiences and those of contemporary European refugee scientists who, despite xenophobia, made enormous contributions to US culture. But the real questions about Tsien's life reside in China.

Chang points out that Tsien's reputation helped to convince authorities that China could build missiles, but what other

factors were involved, and how did Tsien and his colleagues play them? For instance, unlike the United States, where advocates for ballistic missile systems had to compete with a dominant strategic bomber command, China possessed no long-range air force at the close of the Second World War, and so it seems that missile-building started there under very different circumstances. One would like to know how much Tsien played on, and helped to shape, China's Cold War policy for long-range missiles, and whether he relied more on political rather than technical arguments. Chang has left such questions for future study. Her work should act as a stimulus and guide. □

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Geratology comes of age

J. Grimley Evans

Crossing Frontiers: Gerontology Emerges as a Science. By W. Andrew Achenbaum. *Cambridge University Press*: 1995. Pp. 278. £35, \$59.95 (hbk); £12.95, \$18.95 (pbk).

THE history of science is only partly the history of ideas. Ideas have their own dynamic, but social factors, economics and the personality and commitment of individuals can influence their fate. Countries may learn from each other's efforts to develop a social environment in which good ideas can flourish. In its admirable exposition of the history of gerontology in the United States, this scholarly and lucid book will stimulate readers to reflect on the development of the subject, and of science in general, in their own countries.

It is not clear whether the original coining of 'gerontology' (the study of old men) as a malapropism for 'geratology' (the study of old age) was due to illiteracy or latent sexism. Most old people are women, but, although they outlive men, their extra years are mostly years of dependency. The reasons are to be found in evolutionary pressures on cellular and sub-cellular processes in organisms living in the particular social organizations adopted by human and prehuman species. Geratology therefore has firm roots in biology and social science but must justify its existence in the practical world of modern medicine. Nations are ageing; the numbers and proportions of older people in populations throughout the world are rising. The essence and challenge of ageing lie in the loss of adaptability of individual organisms as time passes. This leads in the human species to an exponential increase with age in the risk of death and the prevalence of

disability. Ageing is the product of interactions between extrinsic factors in lifestyle and environment with intrinsic propensities determined by genes and early development. Preventing and alleviating age-associated disability will require collaboration between medical, biological and social sciences.

In Britain, as in several other countries, these three streams in geratology have run in separate channels. As the book's title implies, there have been efforts in the United States to create a unified science of ageing rather than seeing it as a field that several sciences need to cultivate. In Britain, interest in old age was stimulated by the doctors who created the medical speciality of geriatric medicine in the 1950s. They were dealing with the horrors of the old workhouse hospitals, which post-war Britain regarded as no longer acceptable, and their activities were determinedly pragmatic.

Faced with the desperate realities of human suffering, they had little time or leisure to brood on the aetiology and pathogenesis of senescence. Peter Medawar raised the standard of scientific geratology in a 1952 lecture by drawing attention to ageing as a fundamental biological puzzle as well as a social challenge. Sadly, biological geratology in Britain was crippled in the 1960s by retrenchments in Medical Research Council funding. Neglect of older people by a social-work establishment preoccupied with children inhibited the development of a scientific basis for the design and deployment of social services for an ageing population. Political awareness of the sufferings of older people and the economic challenges of an ageing population has been late and remains half-hearted.

In the United States, a scientific approach to the medical aspects of ageing was imported directly from nineteenth-century continental Europe. 'Geriatrics' was a term invented in 1909 by Ignaz Nascher, an Austrian immigrant to the United States. Significantly, Nascher called attention to the poverty of older people and their neglect by social workers as well as to the clinical features of later life. Public concern for the (Unionist) veterans of the Civil War inaugurated a tradition of high-quality medical care for old soldiers that matured into the innovative Veterans Administration programmes — now international pace-setters in health-services research for older people. Needless to say, the path of the Veterans Administration has not always been smooth, as a noisy faction of Americans see in it the unacceptable face of socialized medicine. From the 1920s onward, gerontology was stimulated and supported by private and corporate donors, and this makes for fascinating reading. Some of them may have had bees in their bonnets, but in sparking off powder trails of ideas they had the edge on the anxious jobsworths of public funding. Some of my American friends suggest that Britain owes its lack of similar sparkle from private funding of science to the honours system. Potentially charitable 'fat cats' spend their (or other people's) money on buying knighthoods instead of endowing fellowships or equipping laboratories.

Most striking has been the direct involvement of older people themselves in demanding a better deal from American society. Public lobbying by the Gray Panthers of senators and congressmen was a truly terrifying sight. Letters to the *London Times* have an old-world dignity but are no substitute for the threat of personal violence in encouraging politicians to do the right thing. Tactical voting power of older Americans gave Bob Butler's superb book *Why Survive?* the clout that led to the creation of the US National Institute on Ageing and its multi-million-dollar research budget. Older people elsewhere in the democratic world need to take note.

Despite these enviable achievements, US gerontology is seen in Andrew Achenbaum's final chapter as probably remaining "at the borders of disciplinary enclaves and professional cultures" and as still embodying "too little science". Most readers will join him in seeing a pragmatic and multidisciplinary future for the study of human ageing in the systematic dissection of the genesis of disabilities. This ought to make sense to politicians and the public as well as to scientists. □

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Society and mind

Clive Gamble

The Last Neanderthal: The Rise, Success, and Mysterious Extinction of Our Closest Human Relatives. By Ian Tattersall. Macmillan Publishing USA: 1995. Pp. 208. \$39.95.

The Neanderthal Legacy: An Archaeological Perspective from Western Europe. By Paul Mellars. Princeton University Press: 1996. Pp. 471. \$69.50, £49.50.

WHAT makes the Neanderthals different? For the physical anthropologist, intent on cladistic trainspotting, it is four small hidden features of their skulls rather than the obvious massive brow ridges, low domed crania and powerful, short-legged frames. What often matters to the archaeologist, faced with an abundant legacy of stone tools, are similar small details about how they made these artefacts, rather than the wider aspect of Neanderthal culture, which lacked art, architecture and ritual.

Any account of these 'deep-time' people must reconcile the unglamorous labours of exacting scientific enquiry with the broader enquiry that makes the Neanderthals, and in particular their fate, so fascinating. The minutiae of the specialist must be translated into a context where the stones and bones can determine the contribution of Neanderthals to later evolution. Were Neanderthals replaced by modern-looking people entering their territory in Europe and the Middle East, or is there a seamless continuity between them and the present diverse peoples in this region? Do we look and act like Neanderthals every day of our lives or do we encounter them only in museums?

The title of Ian Tattersall's book leaves us in no doubt about his conclusion. In a richly illustrated and wide-ranging survey, he concludes that we can use Neanderthals as a mirror to ponder our own position in evolutionary history. Such reflection is assisted not only by the excellent colour plates, which present the skulls as art objects, but also by a succinct account of how evolution works and can be applied to the five million years of the hominid fossil record. Tattersall concludes that Neanderthals were different from modern-looking humans in both anatomy and behaviour, but not inferior. Rare for a scientist, he even gives us two short biographies of the last Neanderthal. One, a male, is hunted down by the tall "odd-looking strangers" — a vignette reminiscent of H. G. Wells's extermination of the loathsome Grisly Folk. The other, more in the style of Jorge Luis Borges's parable "The Witness", has a female contemplating her life in the society of the strangers with their clever talk. Tattersall concludes that we will never know in such precise detail what hap-



MATING cuttlefish (*Sepia pharonis*), one of more than 400 colour photographs gracing the informative *Wild Thailand* by Belinda Stewart-Cox and Gerald Cubitt. New Holland, £29.99.

pened but he nonetheless favours the first of these scenarios. What is clear from his book, which extends across that part of the Old World inhabited by the Neanderthals, is the long period of coexistence between the two populations: the last Neanderthal fossils in Zafarraya cave in southern Spain date from some 27,000 years ago — at least 13,000 years after modern-looking humans first appeared in Europe. This is one piece of evidence that allows the author to regard the notion that Neanderthals gave rise to modern humans as totally discredited. Many of its main supporters do not even appear in his index.

Such a firm view is not shared by Paul Mellars. His title refers to the archaeological record rather than to any surviving Neanderthal genes. Instead of covering the Neanderthal world, he concentrates on their heartland in southwest France. His in-depth treatment of the archaeology is led by his study of Neanderthal stone tools, set in the context of how Neanderthals obtained subsistence and raw materials from the region and how they organized their camp sites beneath the limestone cliffs of the Dordogne. He demonstrates that two aspects of the behaviour of Neanderthals make them more similar to us. First, he concludes that variation in their distinctive stone tools corresponds to cultural patterning, where separate long-term traditions of making and doing were fostered by social distance. Second, he argues that during the 200,000 years Neanderthals were

around, their behaviour changed.

Because culture and adaptive change are the archaeological hallmarks of modern humans, Mellars sensibly asks us to re-think the capabilities of Neanderthals and re-assess their difference from the moderns. But when faced with the overwhelming evidence for cultural change in the Upper Palaeolithic revolution, Mellars falls back less convincingly on the idea that complex language made the difference and so led to Neanderthal replacement. Conviction is lacking because, although he devotes chapters to the neglected areas of Neanderthal mind and society, there is less indication of how such themes can be tested with the stone-tool and camp-site evidence. He points archaeologists in typically clear fashion towards the need to overhaul the notion of society and mind as applied to Palaeolithic data. These notions are today locked in their own Neanderthal era and, until we free ourselves, the rich rewards from interdisciplinary research, to which archaeology with its massive databases of past action could contribute, will remain unrealized. A first step would be to harness the discussion of how evolution works from Tattersall's book and apply its principles to the richly detailed Neanderthal legacy where theories of change take second place to the catalogue of difference and similarity. □

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Fight on two fronts

G. E. R. Lloyd

Jewish Thought and Scientific Discovery in Early Modern Europe. By David B. Ruderman. Yale University Press: 1995. Pp. 392. \$30, £20.

SPECULATION about the causes of the so-called scientific revolution continues to pour from the presses and to fill radio and television programmes. But how far is this just idle curiosity? There are fundamental problems with both what has to be explained and how to explain it. Even in the political sphere, 'revolutions' are hard to define and delimit. The import of the term into the history of science may have been pedagogically convenient and it certainly had a role in the claims made for the subject as an academic discipline. But nowadays those who study the complex changes in astronomy, cosmology, optics, anatomy, physiology, pathology and so on in the sixteenth and seventeenth centuries generally agree that to apply the term 'revolution' to cover all of these, or even a determinate subset of them (as in the 'Copernican revolution'), is more misleading than helpful. First, it suggests greater uniformity between and within these fields than we are entitled to assume; and second, it biases discussion towards a focus on discontinuities rather than on the often equally important continuities in the data.

As to which explanatory factors are relevant to the problems once they have been redefined, there too controversy rages. Time was when histories of science were liberally supplied with maps indicating the place of birth of notable figures, although why other men and women of Woolsthorpe, say, did not turn into Newtons was not a problem that received much attention. Is the fact that some such figure was a Protestant or a Roman Catholic relevant? That too has been popular. But most generalizations fall foul of a mass of counter-evidence, for example concerning the Protestants or the Catholics who simply failed to share the views and attitudes deemed important for promoting or inhibiting certain types of study.

This latest study is a mine of information about the ideas, lives and work of particular Jews. Yet despite what the ambiguous title might suggest, this is less a history of the scientific discoveries made by Jews in the early modern period than of Jews' changing attitudes towards such discoveries, or rather towards the study of nature more generally; that is, not so much a study of Jewish contributions to science, which are recognized to have been, in general, inconsequential in the early modern period, as one of the internal polemics within Jewish communities about what to

make of science in the sense of knowledge of the natural world.

Both in time and space the range of the study is daunting. In an introductory chapter, David Ruderman discusses the mediaeval period (roughly the tenth to the fifteenth centuries), although this does not allow much room to comment on such important figures as Maimonides and Gersonides. The core of the book is devoted to three main groups of Jews, those exiled from Portugal and Spain, and those active in eastern Europe and in Italy, from the sixteenth to the early eighteenth centuries.

One recurrent theme is the disputes within the Jewish communities about what to make of the study of nature. Just as Maimonides and Nahmonides took up opposing positions on this in the twelfth and thirteenth centuries, so too were there often bitter controversies, between Moses Isserles and Solomon Luria, and the Maharal of Prague and de' Rossi in the sixteenth century, and between Leone Modena and Joseph ben Judah Hamiz, and between Samson Morpurgo and Solomon Basilea in the seventeenth. In some cases, Jewish defence of the study of nature takes a similar form to some Christian ones, for example in the use of arguments that drove a wedge between science and religion by suggesting that science could claim to deliver no more than the hypothetical and so was no threat to the certainties of faith.

But where Christian apologists fought on one front — with other Christians — many Jews had to fight on two, first with their co-religionists, and then a different

battle to defend their positions in relation to the dominant, Christian, culture. The fact that for most of the period in question Jews were generally excluded from most universities must rank as one of the most important considerations in explaining the nature and limitations of direct Jewish involvement in scientific research. The chief exception, the University of Padua, tends to prove the rule. It did allow Jewish medical students from the early sixteenth century. Many of the author's leaders of Jewish opinion, who often combined the roles of physician and rabbi, graduated from this university: they include Joseph Delmedigo, Hamiz, Morpurgo, Tobias Cohen, David Nieto and Isaac Lampronti.

The main strength of this book lies in the careful study of the lives and writings of these and other figures. But many opportunities are missed. The author notices which texts were originally written in Hebrew, in Arabic, in Latin or in the vernacular. Yet his material cries out for further analysis of the differences that different potential audiences made, of the use of different modes of rhetoric in different contexts, as well as of the social positions, employment and access to patronage of the key figures. The reader is offered some insights into little known aspects of life in Cracow at the time of Copernicus and in Padua in Galileo's youth. But the actual scientific debates against which their work has to be judged are beyond Ruderman's brief. □

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The Psychology of Interrogations, Confessions and Testimony by Gisli Gudjonsson. Wiley, £15.99. Gudjonsson, an eminent forensic psychologist, "reveals the messy truth about interrogation and confession; his welcome and timely book should be required reading for all who strike public attitudes on crime and punishment", wrote Steve Blinkhorn in a review in *Nature* **362**, 655 (1993).

The organization of work in social insect colonies

Deborah M. Gordon

In social insect colonies, workers perform a variety of tasks, such as foraging, brood care and nest construction. As the needs of the colony change, and as resources become available, colonies adjust the numbers of workers engaged in each task. Task allocation is the process that results in specific workers being engaged in specific tasks, in numbers appropriate to the current situation.

Task allocation operates without any central or hierarchical control to direct individuals into particular tasks. The queen does not issue commands, and workers do not direct the behaviour of other workers. We can compare the diverse tasks performed by a colony to the many proteins generated by gene transcription, to the various cell types of a developing embryo, or to the firing patterns of neurons in the brain. What all of these have in common is that, without any central control, individual units (genes, cells, neurons or workers) respond to simple, local information, in ways that allow the whole system (cells, brains, organisms or colonies) to function: the appropriate number of units performs each activity at the appropriate time.

How does task allocation work? What determines, at any moment, which individuals are actively engaged in each task, and how does a colony adjust its effort to the demands of a changing environment? Diverse approaches to these questions include the investigation of the internal states of workers, the sensory physiology of workers' perception of the environment, the development of colony organization as the colony grows older, and the evolutionary pressures on the effectiveness of task allocation. Answers to these questions generally fall along a gradient between two kinds of factors that determine what task an individual worker performs, and when she performs it: internal factors, based on some attribute of the individual, and often considered to be fixed; and external factors, based on some environmental stimulus, and considered to be transient. There are now sufficient data to show that both internal and external factors influence task allocation.

Internal factors

Throughout the 1970s and mid 1980s, research emphasized the internal factors within an individual that determine its task. The idea of a social insect colony as a factory with assembly-line workers, each performing a single task over and over, had widespread appeal. Such a view was consistent with contemporary thinking about analogous systems; examples were the 'one gene, one protein' view of gene action, and the idea that each neuron performs a single function. In social insects, a worker of a given behavioural 'caste' was thought to be intrinsically suited to a particular task, and to perform this task more or less exclusively.

One internal factor associated with task is body size. In some species of ants, colonies include adult workers of two or more sizes. Workers of a particular size might specialize in the task for which their size makes them most suited¹; smaller workers might forage, larger workers might define the nest, and so on. Empirical studies support this for a few species², but polymorphic species, which have more than one size of worker, occur in only a minority of ant genera (44 out of 263); bees and wasps have only one size of worker. Thus polymorphism cannot account for task allocation in most social insects. Other studies show that, regardless of body

size, it is rare for individuals to specialize on a particular task throughout their lives. Although most researchers have moved beyond the idea of division of labour among innate, specialized castes, this idea provided a starting point for the study of task allocation.

A second internal factor associated with task is worker age. It has long been known that honeybee workers move from one task to another as they grow older³. This phenomenon, known as age polyethism, also occurs in some ant species⁴⁻⁶. Young honeybees work inside the nest and older ones forage; juvenile hormone levels influence this transition⁷. In the study of task allocation in honeybees, it was clear from the outset that an individual could not be assigned to a single task on the basis of a static trait such as body size. Instead, much research has examined the internal causes of the predictable progression of tasks performed by a worker as she ages.

Third, genetic factors influence an individual's tendency to perform a task. Honeybee queens mate many times, and selection experiments indicate that worker offspring of different fathers differ in their propensity to engage in certain tasks (though the fathers, as drones, do not perform those tasks)⁸. In one ant species, genotypes differ in this propensity⁹; in others, such individual differences depend both on an ant's early experience and on intrinsic, possibly genetic, variation¹⁰⁻¹². Genetic factors influence age polyethism; honeybees from different patrilineages vary in the rate at which they proceed from one task to the next as they grow older¹³⁻¹⁵.

External factors

Since the mid 1980s, there has been growing interest in how external factors affect task allocation. Task allocation has been shown to be dynamic and labile, in that the number of workers engaged in any given task may continually change.

From day to day, or even hour to hour, an individual worker may perform a variety of tasks¹⁶⁻¹⁸, changing its task as circumstances require¹⁹⁻²¹. (Note that task switching here refers to a more rapid shift in task than those, on the scale of weeks or months, that lead to age polyethism.) An individual may also adjust its activity level, responding to environmental stimuli either by actively engaging in a task or by remaining inactive inside the nest. For example, a honeybee forager's decision whether to collect nectar or remain in the nest depends on how much nectar is already stored in the nest^{22,23}. Such adjustment of activity level in response to environmental stimuli is apparently widespread in social insects²⁴⁻²⁶.

In general, it appears that tasks are interdependent. The number of workers that join in a task at any instant depends on the numbers currently engaged in other tasks. The numbers engaged in various tasks are always changing, owing to individual shifts in task and, on a longer timescale, the birth and death of

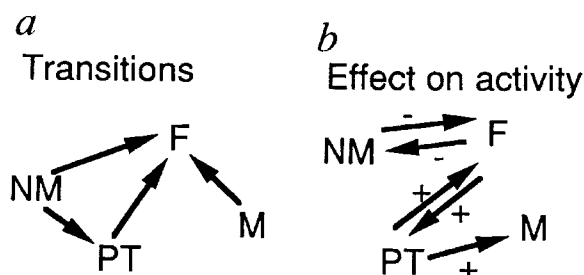


FIG. 1 Interactions among task groups in harvester ants^{20,24,30}. *a*, How individual workers switch tasks when more ants are needed to perform the targeted activity. Arrows show the direction that workers switch tasks. All are one-way only. *b*, How numbers actively engaged in one task affect numbers actively engaged in another. An increase in numbers engaged in the task where the arrow begins has the indicated effect on numbers engaged in the targeted task. F, foraging (searching for and retrieving food); NM, nest maintenance (carrying out soil accumulated at nest entrance during construction of tunnels and chambers); PT, patrolling (scouting for new food sources and responding to disturbances near nest); and M, midden work (sorting the refuse pile).

workers. The mutual relation of numbers engaged in different tasks has been demonstrated dramatically in experiments in which a specific group of workers is removed, for example workers of a certain size²⁷, age cohort²⁸ or task^{29–31}. In response to the removal, the remaining workers shift to perform the tasks previously done by the removed ones. Changes in colony composition also affect age polyethism in honeybees, with individuals proceeding faster from one task to the next if the number of older workers is experimentally decreased^{32,33}; removal of an age cohort also affects age polyethism in some ant species³⁴.

A second demonstration that the activities of different task groups are interdependent comes from perturbation experiments that manipulate worker activity^{20,24,30}. Experiments in the field with colonies of seed-eating ants provide examples of task switching and of changes in activity level (Fig. 1). When extra food becomes available, workers previously engaged in other tasks will switch tasks to forage. When extra clean-up work requires more nest-maintenance workers to be recruited from the reserves inside the nest, the current foragers, a distinct group of workers, are more likely to remain inside the nest. This decision not to forage seems puzzling, but conditions that require extra nest maintenance, such as summer floods that sweep debris onto the nest mounds, may not be ideal for foraging.

Thus individuals constantly alter their task status in two ways: they switch from one task to another, or move between a resting state and the active execution of some task. It is clear that both intrinsic and extrinsic factors contribute to task allocation. Individuals vary in predisposition to participate in certain tasks, and the tendency to perform a particular task changes as the individual grows older. Moreover, these age-dependent predilections are strongly influenced by at least two types of external cues: actions of other individuals, and events in the colony's environment.

There are many analogies with biological systems of other kinds. Genes alter their activity in response to changes in the activity level of other genes; cells in a developing embryo change type in response to the proximity of other cells; and neurons change function in response to signals from other neurons. How can we best understand the regulation of such systems? The next two sections review, first, theoretical models of task allocation, and second, empirical studies of the factors affecting individual task decisions.

Models of task allocation

The growth of theory in social insect research has been inspired by the artificial-intelligence community, who enthusiastically took up the analogy between ant colonies and computational systems

spelled out by Hofstadter³⁵. Many agent-based simulations and their computer-game offspring now have their elements named after social insects. These models have encouraged the development of new ones, based on empirical observation of various aspects of social-insect behaviour³⁶. Here I will outline briefly some models of task allocation, that is, models of processes that could lead to the appropriate numbers of workers engaged in specific tasks.

Recent theoretical work shows how task allocation can operate in the absence of intrinsic differences between individuals. In this work, all individuals are considered to be identical; that is, any individual can perform any task, and an individual's task and activity level are determined by forces external to the individual. The point of these models is to find out how much of the behaviour we observe could arise from interactions between individuals and responses to external stimuli. The models offer an alternative to the view that colony organization arises from the intrinsic properties of individuals.

In some models^{37–39}, task allocation arises solely from interactions among individuals. In one such model, a neural network³⁷, the 'neurons' or units are ants, and an individual's task and activity level depend on the weighted sum of its interactions with others. The results show that even in a system requiring individuals to respond only to simple interactions, the number engaged in one task depends on the number engaged in another.

'Self-organization' models⁴⁰ are another example of models based on interactions between individuals. If individuals tend to follow others with whom they interact (for example, by sensing a pheromone deposited by the other individuals), random movement can coalesce into spatial patterns such as foraging trails. These models have been applied mostly to describe the formation of foraging trails by ants^{41,42}, with recent application to other tasks²⁹.

The 'foraging-for-work' model^{43–45} differs from neural network and self-organization models in that environmental stimuli are more important than interactions among individuals. An individual's decision whether to engage in a task depends on whether it finds itself in a location where execution of that task is required. Extrinsic factors do not fully determine an individual's task because unspecified factors, possibly intrinsic differences among individuals, may determine each ant's location⁴⁶.

Some models refer both to environmental stimuli and to interactions among individuals^{23,47,48}. One such model⁴⁷ is general in the sense that it describes the allocation of workers either to different tasks, or to different aspects of one task (for example, foragers to different resources). The model predicts a colony's ability to track and respond effectively to a changing environment by showing how quickly the colony can allocate the optimal distribution of workers to tasks or resources. The results show how a colony's response to its environment depends on the number of workers, the rate of information transfer among them, and whether task performance generates negative feedback for further execution of the same task (Fig. 2).

I know of no formal models of the dynamics of task allocation based primarily on intrinsic factors. However, the discovery of genotypic differences in the behaviour of honeybee patriline has led to the following informal model⁴⁹ (and another is described below³³). Suppose each genotype has a threshold stimulus at which the bee will engage in a task. At low levels of stimulus, the task will be accomplished by the individuals with a low threshold; at high levels of stimulus, individuals of other genotypes, that normally perform other tasks, will switch to respond to the high stimulus. So far, empirical studies support the threshold model with regard to some tasks but not others^{50,51}.

What determines an individual's task?

Individuals decide which task to perform, and whether to engage in it actively in a given situation. Social insects communicate mostly through chemical and tactile cues. What determines an individual's task and activity level?

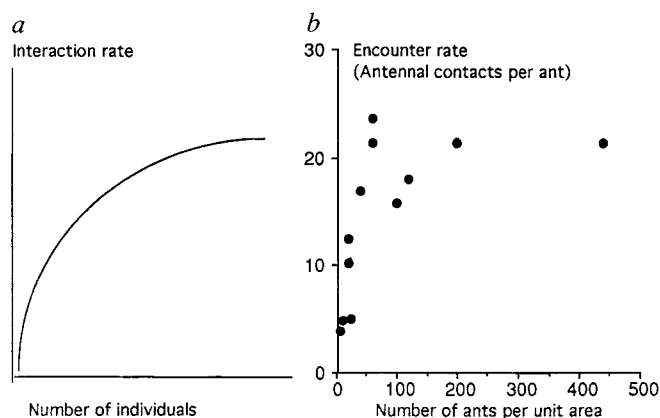


FIG. 2 Theoretical and observed relation of interaction rate and density. *a*, Theoretical prediction of optimal rate of information transfer⁴⁷. In the model, an individual's task is determined by the environmental stimuli it encounters (for example, foragers' encounters with food), and by the rate at which it interacts with successful individuals engaged in the same and other tasks. Regulating interaction rate at high density prevents excessive numbers of individuals from performing a task. *b*, Observed relation of interaction rate and density⁵⁷. Interaction rate was measured as the number of antennal contacts per ant, over a range of densities (number of ants per unit area), in the ant *Lasius fuliginosus*. Ants regulate interaction rate at high densities.

The results summarized above show that individuals respond to changes in the number of individuals engaged in some other task. Because no insect is capable of counting, or understanding the whole pattern of colony behaviour, no individual can communicate a global assessment to the others such as "Hey, you! There are now more than 35 ants over there doing urgent nest maintenance so stop foraging!" Because individuals move around, and many pheromones are highly volatile, the relevant cues are unlikely to be based on the quantity of pheromone accumulated over a long time in one place. Instead, each individual probably responds to local, transient cues that reflect the global situation.

In some cases, individuals respond to cues based on the ways that others have modified the environment through their work. The construction of the paper nest by colonies of *Polybia* wasps provides examples of this^{25,48,52-54} (Fig. 3). A forager's decision whether to collect more building material (wood pulp) depends on how long she had to wait before a builder accepted her last pulp load; this in turn depends on how much pulp is currently available to nest builders.

Individuals may also respond to the rate of interaction with others engaged in like or unlike tasks. Workers would have to recognize the task of other workers they meet, perhaps by chemical cues associated with particular tasks (for example, foragers might carry the odour of food)⁵⁵. Interaction rate, or the interval between successive encounters, depends on the total number of individuals present per unit area. Thus each individual need only keep track of the interval between its encounters with others to monitor changes in the density of the whole group.

Several lines of evidence support the hypothesis that encounter rate can influence the task decisions of individuals. Task allocation depends on colony size in ants, because the effect of one task group's activity on that of another task group varies with colony size^{24,56}. Interaction rate also depends on colony size, as the more individuals there are per unit area, the more encounters each one will experience. Ants can regulate encounter rate; one ant can perceive another at a distance and will avoid excessive encounters at high ant densities⁵⁷. Theoretical work shows that this allows a large colony to match its efforts to environmental changes⁴⁷ more effectively (Fig. 2). The role of interaction rate in task allocation remains an intriguing area of investigation.

We know that intrinsic factors influence what task an individual

does, because task is sometimes correlated with age, genotype or both. We also know that extrinsic factors affect task performance, because colonies respond in a multitude of ways to changes in the environment and in the current distribution of workers among different tasks. However, we know very little about the interaction of the two, that is, how an alteration of internal state affects an individual's response to external stimuli⁵⁸, or how environmental stimuli can trigger an individual's internal state to change. For example, young honeybees will move more quickly into foraging tasks, typically performed by older bees with high levels of juvenile hormone, if old bees disappear from the colony. It seems that interaction between old and young bees inhibits biosynthesis of juvenile hormone in younger bees, and, when older bees are removed, biosynthesis of juvenile hormone is increased³³. Thus an extrinsic factor (colony composition) somehow influences a physiological factor (the level of juvenile hormone) and this relation is mediated by interactions among workers. The relations of internal to external factors that influence an individual's task decisions still require further study.

The evolution of task allocation

A colony's survival and reproduction depend on how well it can match its efforts to the challenges of a variable environment. The evolution of task allocation could be seen as a process that generated a set of dynamical rules that determine how much a task will be performed in each ecological situation.

Questions about how task allocation systems have evolved are inseparable from questions about how such systems work. If an individual's task depends entirely on some fixed and innate attributes of the individual, the evolution of task allocation could be seen as a process that led to individual differences that cause certain individuals to perform the right task at the right time. Early work on the evolution of task allocation was based on the idea of division of labour among specialized individuals. Natural selection would shape the distribution of specialized individuals in a colony to provide the requisite number of individuals to perform each task¹. Thus, for example, if a colony's reproductive success is increased by abundant food, natural selection might favour a high proportion of workers that specialize in foraging.

Individuals switch tasks, however, so the evolution of task allocation systems cannot be merely the production of distinct types of individuals, each suited to a particular task. Instead, there may have been natural selection for individuals to switch from one task to another in the most ecologically appropriate way. For example, when a new food source suddenly becomes available to a harvester ant colony, which competes with other seed-eating species for food⁵⁹, ants previously engaged in other tasks will

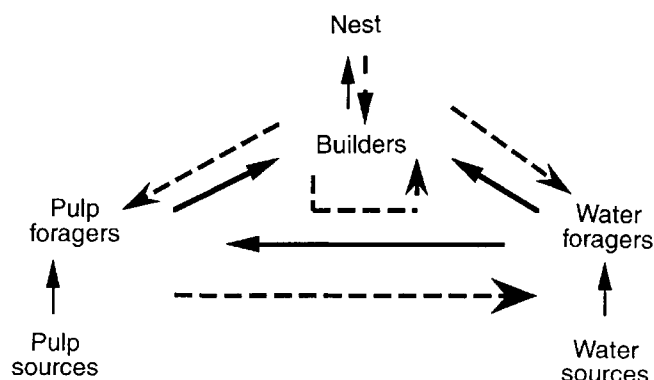


FIG. 3 Flow of information and materials among task groups during nest construction by the wasp *Polybia occidentalis* (modified from ref. 25). Solid lines represent direction of flow of nesting materials, water and wood pulp; broken lines represent direction of information transfer through interaction of workers.

switch to foraging (Fig. 1) allowing colonies to take advantage of sudden increases in food availability. There may have been selection for a propensity in any outside worker to switch to foraging when a new food source appears, rather than selection for higher numbers of specialized foragers.

Individuals decide whether or not to perform a task actively, and this influences the extent to which a task is accomplished. Selection might be expected to shape the processes that determine when individuals are active. For example, harvester ant foragers reduce their activity from one hour to the next when the colony's rate of food intake declines⁶⁰, which prevents foragers from spending energy to forage when food availability is low. Genotypic variation among honeybee patrines suggests that natural selection could act on task allocation through queens' mating decisions. If the progeny of different honeybee fathers differ in propensity to accomplish a task, the distribution of patrines in a colony would influence its ability to respond to its environment⁶¹.

The study of the evolution of task allocation is still in its infancy. With some important exceptions⁶²⁻⁶⁶, most effort is devoted to understanding how task allocation operates in particular species, and until we know more about what kinds of task allocation systems have evolved, we will not be able to say much about how they evolve. Although there has been considerable theoretical work on the evolution of the partitioning of reproduction among individuals—who lays the eggs, and which sex is produced—this does not address the question of how workers are allocated to day-to-day colony tasks.

The evolutionary ecology of task allocation could be investigated empirically. We could examine how variation among colonies in task allocation is related to variation among colonies in reproductive success. Such studies could provide new insight into the evolution of other systems of interacting or cooperating units that function without central control.

Conclusion

The behavioural ecology of social insects has only recently begun to generate a variety of approaches and attract interest from many different disciplines. So far only a tiny fraction of social insect species have been studied. Social insects obtain food, build nests, and defend their colonies in an astounding variety of ways. We will probably discover the same diversity in the ways that these tasks are regulated. The data show that some combination of the internal and external factors outlined above contribute to individual decisions about task performance, and eventually we may abandon the dichotomy between internal and external causes. Current theoretical work far outreaches the data, but the models show that task allocation could operate even in a colony of identical individuals, if regulation is mediated by local cues that reflect the numbers of workers performing a task and the extent to which the task is accomplished. □

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ACKNOWLEDGEMENTS. I thank M. Brown, B. Crow, P. Green, J. Gregg, K. Human, S. McConnell, D. Wagner and J. Weimann for comments on the manuscript.

Volcanic growth faults and the origin of Pacific abyssal hills

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The topographic features known as abyssal hills characterize >30% of the ocean floor, and yet their origin has been the subject of vigorous debate for over 40 years. Submersible-based investigations show that Pacific abyssal hills are created on the flanks of the East Pacific Rise as horsts and grabens which lengthen with time. Hills are bounded on one side by ridge-facing scarps produced by normal faulting, and on the other by more gentle slopes produced by volcanic growth faulting.

ALTHOUGH abyssal hills are unspectacular when compared to terrains created by continental collisions, subduction-related processes or hotspot volcanism (for example, the Himalayas, the Andes and the Hawaiian islands, respectively), abyssal hills are nevertheless the most abundant geomorphic structures on Earth. In the Pacific Ocean, and in other ocean basins formed by fast-spreading ridges, the abyssal hills are typically 10–20 km long, 2–5 km wide and 50–300 m high, are oriented approximately perpendicular to the spreading direction, and cover virtually all of the ocean floor except where they are buried beneath sediment^{1–4}. Dietz⁵ first suggested in 1961 that abyssal hills are created by a combination of “intrusion and extrusion and then placed under rupturing stresses as the sea floor moves outward”. Ever since then, lively debate has ensued over whether the abyssal hills of the Pacific are created on the spreading axis or at some distance off-axis, and whether the hills owe their relief and shape primarily to volcanic constructional processes, faulting, or some combination of the two (Fig. 1) (see, for example, refs 3 and 6–20).

In the past decade, acquisition of high-resolution acoustic data has focused the debate over the origin of the Pacific abyssal hills. High-resolution profiles across abyssal hills were collected in a number of locations across the flanks of the East Pacific Rise (EPR) using a narrow-beam echo-sounder on the Deep Tow system. These profiles revealed that the hills are bounded by steep slopes facing both towards and away from the axis of the EPR^{11,13,21}. These results suggest that inward- and outward-facing slopes of the abyssal hills are the product of normal faults which have created high standing horsts. The swath maps produced by multibeam sonar systems have lower slope resolution than the Deep Tow profiles, but multibeam systems provide continuous coverage bathymetric maps and high-fidelity images of sea-floor features in plan view²². Multibeam bathymetric maps reveal that the abyssal hills flanking the EPR have a distinctive asymmetric shape¹⁸. The side of the hill facing the axis of the EPR is usually a steep slope that is continuous and straight along the length of the hill. In contrast, the side of the hill facing away from the ridge axis is typically less steep, is often sinuous rather than straight along-strike, and is decorated with lobate landforms. This asymmetric shape may arise from the interplay of two processes: faulting and volcanic construction (Fig. 1)^{7,9,11,13,23}. To determine the relative importance and timing of these processes and to advance our understanding of abyssal hill formation, direct near-bottom imaging

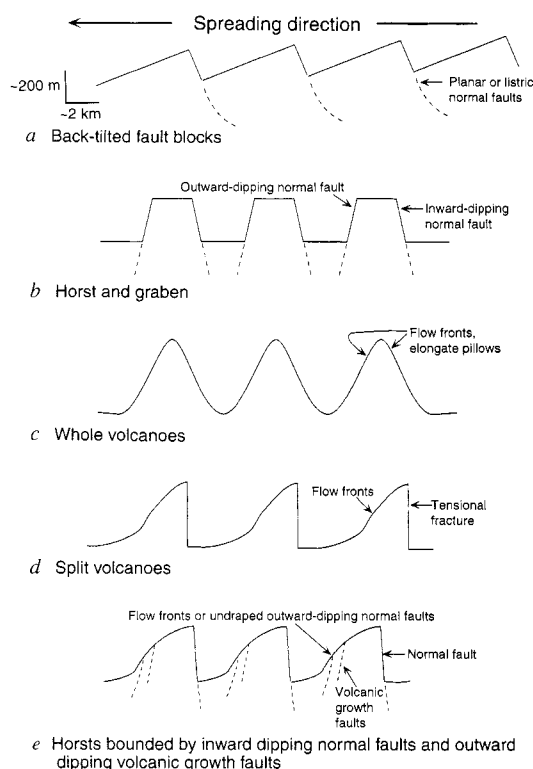


FIG. 1 Five models for the development of abyssal hills. *a*, Back-tilted fault blocks (episodic inward-dipping normal faulting off-axis); *b*, horst and graben (episodic inward- and outward-dipping faulting off-axis); *c*, whole volcanoes (episodic volcanism on-axis); *d*, split volcanoes (episodic volcanism and splitting on-axis); *e*, horsts bounded by inward-dipping normal faults and outward-dipping volcanic growth faults (episodic faulting off-axis and episodic volcanism on- or near-axis).

of the inward- and outward-facing slopes of abyssal hills was required.

Here we report observations made with the submersible *Alvin*, which lead to a model for the formation of Pacific abyssal hills. We find that they are created ~2–6 km away from the axis of the EPR, and are bounded by normal faults dipping towards the axis

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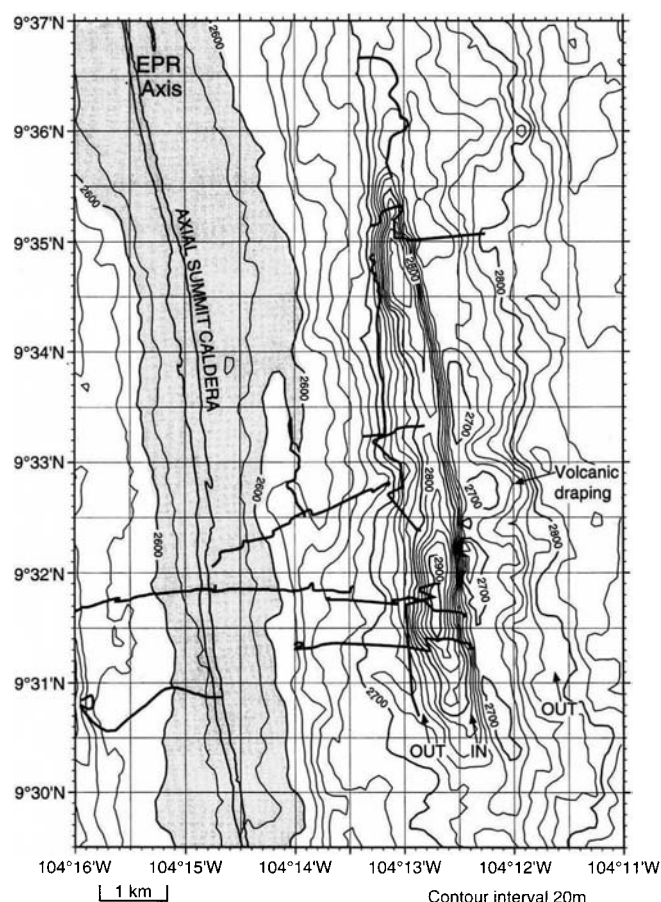


FIG. 2 Detailed Sea Beam bathymetry (depths <2,600 m shaded) of a near-axis trough showing dive tracks (thick lines) and the location of axial summit caldera from ref. 34. "In" and "out" show examples of slopes which face in (towards) or outwards (away from) the spreading axis. "Volcanic draping" shows one location of the morphology produced by draping of an outward-facing scarp by syntectonic lava flows.

and 'volcanic growth faults' dipping away from the axis. The repeated draping of fault scarps that face away from the spreading axis by syntectonic near-axis lava flows results in a volcanic growth fault whose surface exposure appears to be volcanic, but whose vertical relief is almost entirely faulted in origin. Activity on these outward-dipping volcanic growth faults diminishes ~6 km off-axis. In contrast, inward-dipping faults continue to be active at least 30 km off-axis and produce further growth in length and height of abyssal hills. For fast-spreading centres, the end product is an asymmetric abyssal hill.

Abyssal hill controversy

Independent of the model adopted to explain the relief and shape of the abyssal hills flanking the EPR, it seems clear that the hills are not formed along the axis of the EPR, because almost nowhere along the axis is significant relief created either by faulting or volcanic processes²⁴. The axial high itself is not evident on the flanks and seems to disappear off-axis, suggesting that it is created primarily by the buoyancy of hot rock and magma beneath the rise²⁵. Indeed, seismic layer 2A (interpreted to be the volcanic layer)^{26,27} is thinnest on the spreading axis and doubles in thickness only 1–4 km off-axis^{27,28}. The added 150–200 m of lava is more than enough to inundate even the largest axial summit calderas imaged along the axis of the EPR, so the rare occurrences of significant relief on-axis²⁹ are not preserved off-axis²⁵.

The first development of terrain that has the relief and size of an abyssal hill is located several kilometres off-axis on the flanks of

the EPR axial high, where narrow troughs are observed on high-resolution maps of both the northern^{22,30} and southern EPR^{31,32}. These troughs are aligned discontinuously along the flanks of the EPR, range in length from a few kilometres to greater than 30 km, and develop relief of tens of metres to over 200 m (Figs 2, 3a). Based on an analysis of multibeam maps, the origin of the troughs was ambiguous and they have remained unexplained. Whereas the steep and straight axis-facing slopes of the troughs are suggestive of fault-controlled scarps, the outward-facing slopes are not as steep or straight and are often associated with small (10–50 m high) conical structures suggestive of volcanism. Our observations indicate that it is the deepening, lengthening and linking of these troughs as a function of time which defines the intervening abyssal hills; the origin and tectonics of these troughs are therefore crucial to the question of abyssal hill origin.

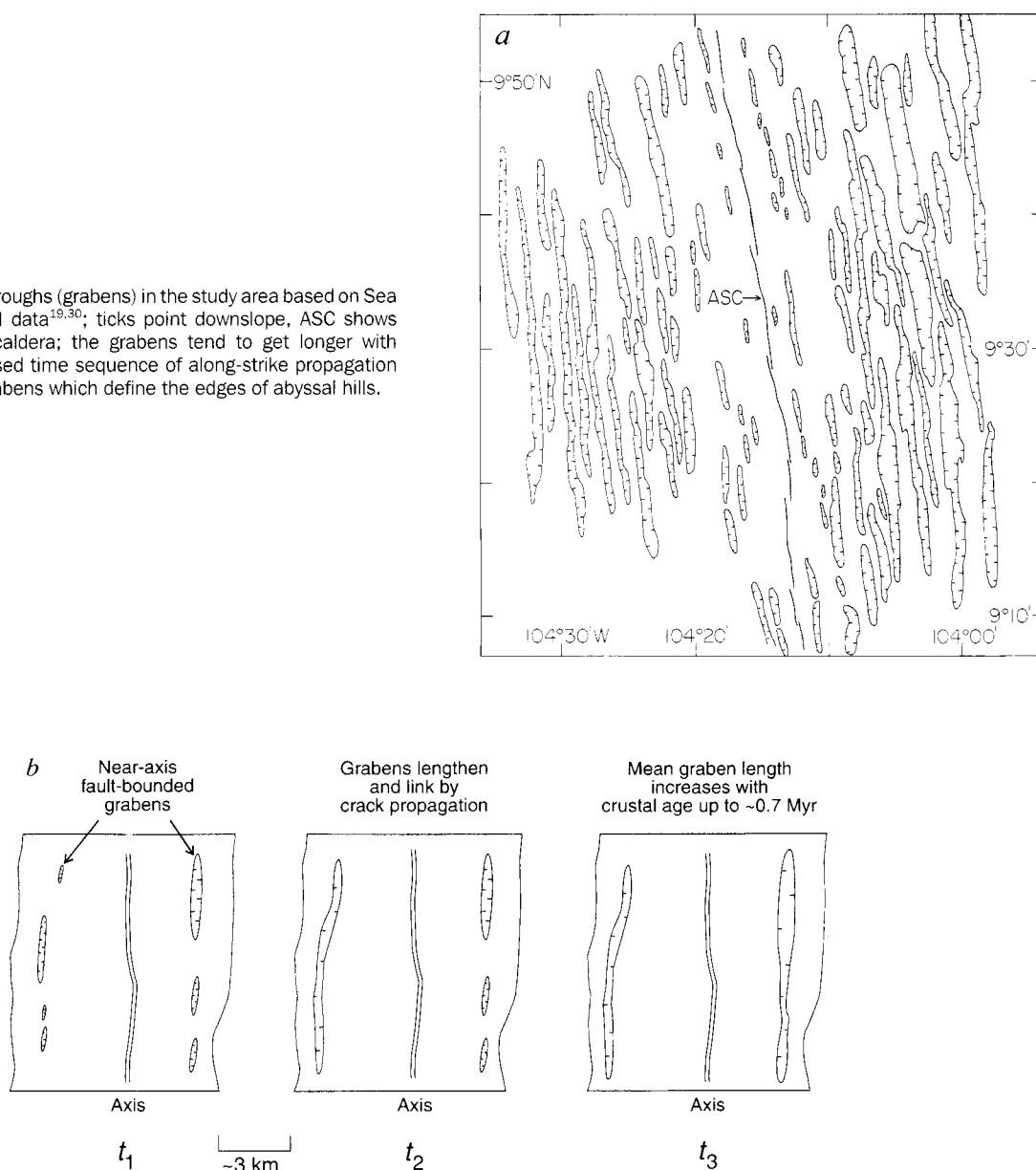
Alvin investigations of abyssal hills

To investigate further abyssal hills and troughs, we conducted a 17-dive programme with the submersible *Alvin* on the fast-spreading EPR (109 mm yr⁻¹; ref. 33) between 9° and 10° 30' N (U.S. Office of Naval Research Natural Laboratory). The relevant data sets collected from *Alvin* include continuous video coverage from forward- and downward-looking cameras, nearly continuous 35 mm still photographic coverage, and mesotech sonar 150-kHz, super-high-resolution swath bathymetry. Our field investigations spanned fully mature hills on crust ~0.7 Myr old to those in the initial stages of development on the flanks of the spreading axis in the zone of trough development. Dives on even older hills were not considered owing to the obscuring effects of sediment cover.

Mature hills. We focused our observations on features that can be used to distinguish between the different models proposed to explain abyssal hill formation (Fig. 1). For the six hills studied using *Alvin*, the sides of hills which face the spreading axis are found to be faulted escarpments, as evidenced by: exposures of sharply truncated pillow lavas, steepness of slopes (60°–90° based on mesotech profiles and direct observations), rectilinearity of scarps when traced along strike, and the abundance of fresh, well sorted talus at the bases of scarps (in contrast to the poorly sorted whole pillow talus that occurs at the bases of flow fronts)¹³. None of the inward-dipping faults exhibit the glassy cooling ledges of a subsiding lava lake which are so common along the walls of the axial summit caldera^{34,35}. This offers support for the idea that inward-dipping faults are not fossil axial summit caldera walls. We also observe that the tops of the hills are within a few degrees of horizontal, based on fine-scale mesotech bathymetry, and constrained by observations of horizontal cooling ledges on lava pillars³⁶ in a fossil lava lake at the top of a hill 6 km off-axis. Based on these observations, the 'back-tilted fault block' and 'whole volcano' hypotheses are eliminated (Fig. 1). On the slopes of mature hills that face away from the axis, we observe intact lava flows (primarily elongate pillows), which are consistent with either the split volcano or horst/graben hypotheses.

Birth of hills. After establishing the asymmetric character of fully developed abyssal hills located on crust up to 35 km off-axis, we focused on a few selected troughs, located 2–6 km off-axis, to investigate the initiation of abyssal hill development (Figs 2, 4a). As with the mature abyssal hills, the inward-facing slopes of the troughs are fault-controlled scarps with relief of 10–60 m, whereas the outward-facing slopes are dominated by elongate pillow lavas. On along-strike traverses of the outward facing slopes, we crossed well sorted fault talus every few hundred metres. Manoeuvring the submersible uphill from these talus ramps, we encountered faulted scarps 5–25 m high, dipping 60°–90° away from the spreading axis. At the tops of the scarps are fresh elongate pillow lavas in a frozen cascade over the near-vertical scarps of sharply truncated pillow lavas. Travelling north and south along strike (Fig. 4a), we found fault scarps which include those that are entirely exposed; partly exposed with some sections covered by elongate pillows; or entirely draped by lavas flowing from the

FIG. 3 a, Summary map of troughs (grabens) in the study area based on Sea Beam^{22,30} and Sea MARC II data^{19,30}; ticks point downslope, ASC shows location of axial summit caldera; the grabens tend to get longer with distance off-axis. b, Proposed time sequence of along-strike propagation and linkage of near-axis grabens which define the edges of abyssal hills.



direction of the spreading axis (demonstrating that the source of the lava flow was either the axis itself or volcanic centres within 2–3 km of the axis). Many of the draping elongate pillows have pinched-off and elongate pillow sections, and tubes have fallen ~10 m to the base of the scarps.

In a few cases, faults could be traced to their termini along strike. The fault relief decreases towards the ends of the faults. Where deformation does not relay to neighbouring *en echelon* faults, the faults invariably terminate in fissures 1–3 m wide which narrow to closure within ~10 m along strike. The ends of exposed faults are not characterized by lava burial or by warping of overlying lava flows.

On 13 dives (including 4 from earlier work in the area³⁷) we studied 3 near-axis troughs centred at 1.5, 2.5 and 5 km away from the axis. The troughs appear to be successively more developed (that is, exhibit greater relief and along-strike continuity) as a function of distance away from the spreading axis. The maximum depths of the troughs increase away from the axis, from 30 to 100 to 200 m respectively, while the lengths of the troughs increase from 3 to 6 to 11 km respectively. A significant length (18 km) of zigzag traverses along and up and down the outward-facing slopes of these three near-axis troughs

show the same pattern of exposed active fault scarps partially draped by elongate pillow lavas.

Volcanic growth faults. The outward-facing slopes of the hills are neither simple outward-dipping normal faults, as would be predicted by the horst/graben model, nor are they entirely volcanic-constructional, as would be predicted by the split volcano model. The near-axis troughs, which define the edges of embryonic abyssal hills, develop 2–6 km off-axis in a “flanking tectonic province”, instead of developing directly on the spreading axis as the split volcano model (proposed for intermediate spreading ridges) would predict^{9,23}.

We interpret the outward-facing slopes as volcanic growth faults (Fig. 4b). Small scarps produced by episodes of normal faulting are buried near the axis by syntectonic lava flows originating either at the axis or between the flanking tectonic province and the axis (that is, within ~2–3 km of the axis; see, for example, refs 37 and 38). Repeated episodes of dip-slip faulting and volcanic burial result in structures resembling growth faults, except that the faults are episodically buried by lava flows rather than being continuously buried by sediment deposition. An important difference between volcanic growth faults and traditional sedimentary growth faults is that occasionally enough dip-slip offset may

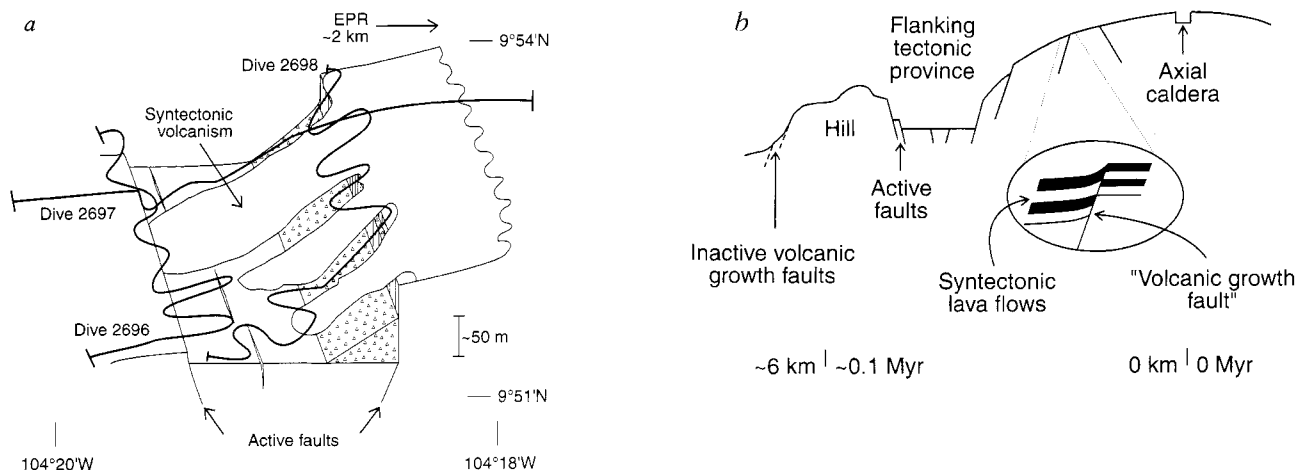


FIG. 4 Volcanic growth faults; a, lavas cascading over outward-facing scarps based on dives in the 9° 54' N near-axis graben; b, cross-sectional depiction of the development of volcanic growth faults.

accumulate (> 10 m) between episodic volcanic eruptions so that pillow lavas which flow over the scarp elongate to the point of necking (Fig. 4a). This failure to drape the scarp completely and continuously leaves sections of the fault scarp exposed for some period of time. Based on these observations, the abyssal hills are horsts and the intervening troughs are grabens, with the important modification to the horst/graben model that the outward-facing slopes are created by volcanic growth faulting rather than traditional normal faulting. Thus, volcanism is a more important aspect of the morphology of abyssal hills than previously appreciated in the horst/graben model.

Multibeam bathymetric data and side-scan sonar records support the interpretation that outward-dipping volcanic growth faults accumulate nearly all of their throw within ~ 6 km of the axis (within the flanking tectonic province, Fig. 5). These faults are thus near enough to sources of volcanism, either the neovolcanic zone or near-axis sources, to be subject to burial throughout their development^{39,40}. In contrast, inward-dipping faults act as tectonic dams to lava flows rather than being buried by them^{39,40}. They continue to be active > 30 km from the spreading centre in this area^{41,42} resulting in continued growth (increase in length and height) and modification (for example, minor outward tilting of 2° – 3°) of some of the hills off-axis (Fig. 4).

Lengthening of hills. To understand the temporal and spatial interplay of volcanism and tectonism in the development of abyssal hills along the axis of the fast-spreading EPR, it is necessary to consider the process from a plan-view perspective (Fig. 3a). We observe that grabens which define the edges of abyssal hills lengthen as a function of distance off-axis (Fig. 5, top). If the process is relatively steady-state, this means that they lengthen as a function of time. We propose that the near-axis grabens behave as an array of cracks, lengthening and linking via crack propagation along-axis as the lithosphere is stretched within the plate boundary zone⁴³ (Fig. 3b). In this way, abyssal hills begin as embryonic horsts and grabens that develop in height and length as a function of time and distance from the spreading axis. The integrated result of these processes is an asymmetric abyssal hill bounded by a steeply dipping normal fault on the side facing the spreading axis, and bounded by a volcanic growth fault on the opposing side.

Other types of abyssal hill

This process describes the origin of most abyssal hills at fast-spreading centres characterized by an axial high. At slow- to intermediate-rate spreading centres and adjacent to ridge-axis discontinuities, other processes are likely to be important. Near ridge-axis discontinuities on fast-spreading centres, the lithosphere appears to be sufficiently thick to support axial volcanic constructions²⁵. Subsequent propagation and decapitation⁴⁴ of the

ends of spreading segments results in the rafting-off of whole volcanoes as abyssal hills (Fig. 1c). At intermediate-rate spreading centres, abyssal hill structure may vary with the local magmatic budget. Where the budget is starved and the axis is characterized by a rift valley, abyssal hills are generally back-tilted fault blocks¹².

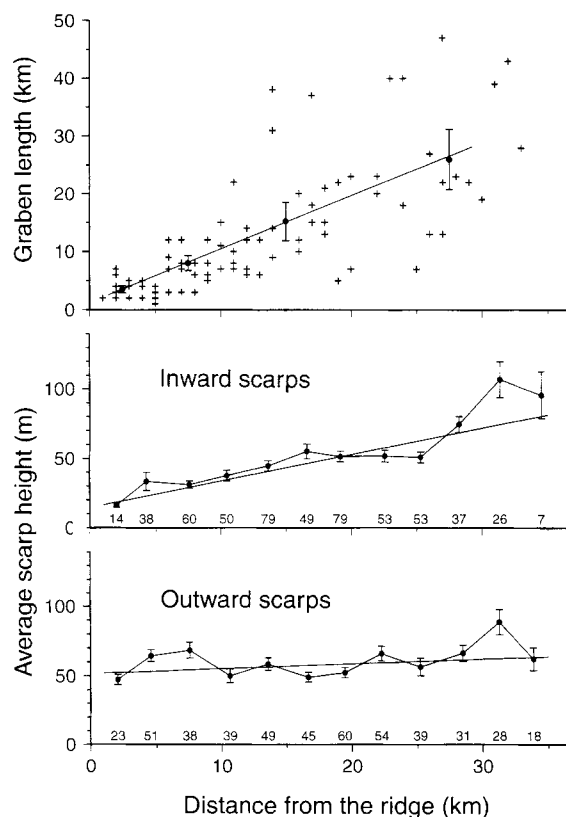


FIG. 5 Top, graben length versus distance away from the spreading axis. Dots show average graben length in bins 0–5 km, 5–10 km, 10–20 km and 20–35 km off-axis, error bars are ± 2 standard errors, the line is a linear regression. Bottom, average scarp heights (facing inwards or towards the spreading axis, and outwards or away from the axis) as a function of distance from the axis; numbers indicate how many scarps were tabulated within each 3-km-wide bin extending from 9° 20' N to 9° 50' N, error bars are ± 2 standard errors, straight lines are linear regressions; for outward-facing scarps, $R = 0.11$, for inward-facing scarps, $R = 0.90$. Inward-facing scarps show continued growth to the edge of the study area (also indicated by seismicity⁴⁵) perhaps beyond; for outward-facing scarps, growth diminishes within ~ 6 km of the spreading axis.

Where the magmatic budget is robust and an axial high is present, the axial lithosphere is episodically thick enough to support a volcanic construction which may then be split in two along the spreading axis, resulting in split-volcano abyssal hills^{9,25} (Fig. 1d). At slow-spreading centres characterized by an axial rift valley,

back-tilted fault blocks and half-grabens may be the dominant origin of abyssal hills (Fig. 1a), although there is continued controversy over the role of high- versus low-angle faults, listric faulting versus planar faulting, and the possible role of episodes of volcanism^{17,45,46,47}. □

Received 11 August 1995; accepted 26 January 1996.

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ACKNOWLEDGEMENTS. We thank diving scientists D. Scheirer, C. Fleutelot and N. Beedle for their assistance; M. Perfit and D. Fornari for use of observations from a previous dive programme; J. Karson, J. Goff and R. Haymon for reviews; A. Padgett for graphics; and the *Alvin* group, and the captain and crew of *Atlantis II* for their expertise. This work was supported by the US Office of Naval Research.

A light-entrainment mechanism for the *Drosophila* circadian clock

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Biochemical studies indicate that the *Drosophila* timeless protein (Tim) is a stoichiometric partner of the *period* protein (Per) in fly head extracts. A Per–Tim heterodimeric complex explains the reciprocal autoregulation of the proteins on transcription. The complex is under clock control, and many circadian features of the Tim cycle resemble those of the Per cycle. However, Tim is rapidly degraded in the early morning or in response to light, releasing Per from the complex. The Per–Tim complex is a functional unit of the *Drosophila* circadian clock, and Tim degradation may be the initial response of the clock to light.

CIRCADIAN rhythms have been characterized in many eukaryotes and prokaryotes. They are generated by an endogenous clock, or pacemaker, which runs under constant conditions but is normally entrained by the daily light–dark cycle. Despite decades of intense study, there are few molecular studies that indicate how circadian clocks function, neither have many clock components been identified^{1–4}.

In *Drosophila*, the *period* (*per*) gene product makes an important contribution to circadian rhythms and encodes a likely state variable of a circadian pacemaker^{1,3,5}. Mutations in *per* either shorten (*per^S*), lengthen (*per^L*) or abolish (*per⁰*, a null mutation)

circadian rhythms⁶. Also, *per* messenger RNA and *per* protein (Per) undergo circadian fluctuations in levels^{7–9}. As *per^L* and *per^S* mutants alter behavioural rhythms and transcriptional cycling in parallel, it is likely that the clock includes a feedback loop in which Per activity affects its own transcription^{7,10}. Various post-transcriptional mechanisms have also been proposed to regulate Per levels and activity in a circadian manner^{11,12}, including phosphorylation⁹, nuclear transport^{13,14} and protein stability^{9,15}.

The gene *timeless* (*tim*) is a recently identified second clock gene of *Drosophila*, and it shares many features with *per*, including mRNA cycling^{16,17} (for reviews, see refs 18,19). Like the *per⁰* mutant, the null *tim⁰* mutant shows no behavioural or molecular rhythms^{17,20}. The *tim* protein (Tim), as well as Per, is likely to be

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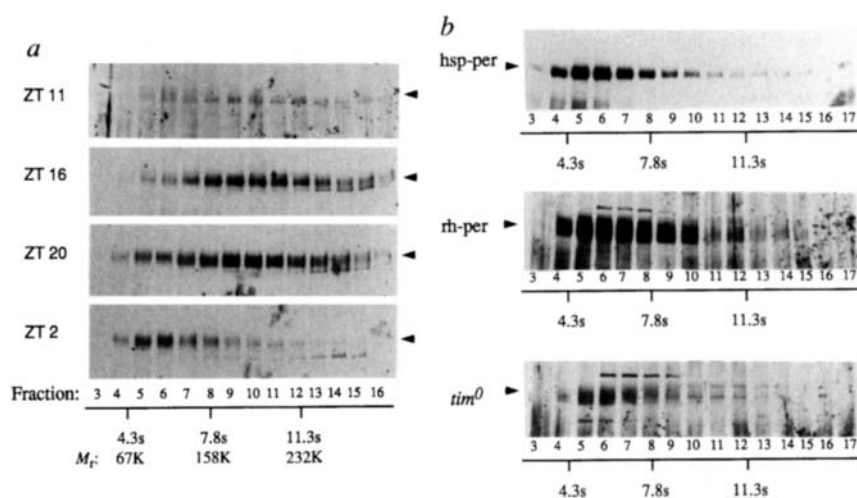
FIG. 1 Sucrose gradient analyses of Per complexes. **a**, Temporal changes. Protein extracts of wild-type fly heads from ZT11, 16, 20 and 2 were run on 5–25% sucrose gradients. Gradient fractions were assayed by western blotting with an anti-Per antibody. **b**, Per sediments around fractions 5–6 (5.3 s) in transgenics *hsp-per* (*hspcper-23a*, heat shocked at 37 °C for 1 h and recovered for 2 h) and in the *tim⁰* strain. Shown below the fraction numbers are the positions of protein standards: albumin (M_r 67K, 4.3 s), aldolase (M_r 158K, 7.8 s) and catalase (M_r 232K, 11.3 s). Arrowhead, Per. Other bands (all appearing thinner than Per) are nonspecific, including the two in the *tim⁰* panel that are of similar size to Per but peak in fractions 9–12. Note that much less extract was used for *hsp-per* than for the others (see Methods).

METHODS. Fly head extracts were prepared as described⁹ with minor modifications: 20 mM β -glycerophosphate and 100 μ M Na_2VO_4 were added to extraction buffer. Extract (40 μ l, except for *hsp-per* which was 5 μ l extract plus 35 μ l extraction buffer) was layered on top of a 12-ml, 5–25% sucrose gradient in extraction buffer. After centrifugation at 40,000 r.p.m. for 12 h in an SW40Ti rotor, 400- μ l fractions were collected from the top down. The fractions were then precipitated with 10% trichloroacetic acid (TCA) and resolved on 6% (29:6:0.4) SDS-PAGE.

involved in the transcriptional feedback regulation of both genes¹⁷. Moreover, there are indications that Tim participates in some post-transcriptional regulation of Per, including nuclear translocation and protein stability^{13,15}. Because Per can associate with Tim *in vitro* and in yeast²¹, some of this circadian post-transcriptional regulation may involve direct contact between Per and Tim. However, Per-interacting proteins have not been identified in fly extracts.

Per is in a large protein complex

To search biochemically for Per-interacting proteins, we first assayed by sucrose gradient centrifugation the size of Per complexes in extracts from flies collected at different times of a 12 h:12 h light:dark (LD) cycle. As shown in Fig. 1a, at Zeitgeber

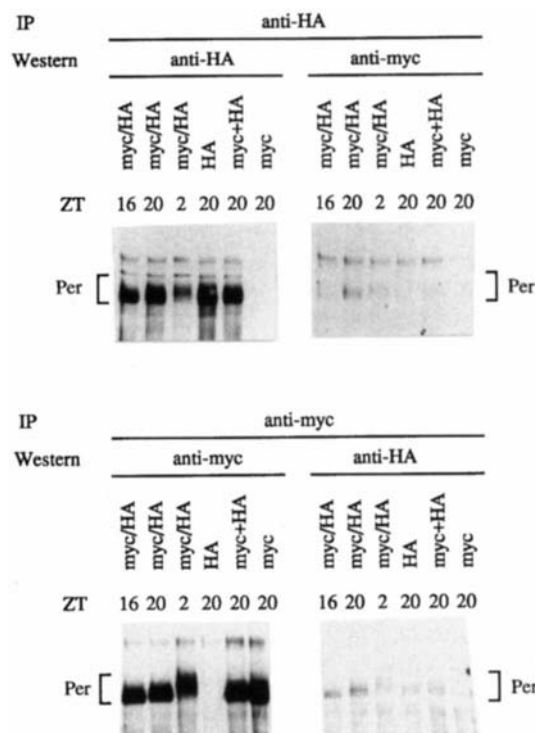


Western blotting with anti-Per was visualized by ECL (Amersham). The M_r of the large complex was estimated by measuring the Stokes radius by gel filtration on a Superose 6 (Pharmacia) column (data not shown). This value, ~ 90 Å, taken together with the sedimentation coefficient of 9.1S (Fig. 1), gives rise to an estimated M_r of 337K for the large complex³⁴.

times (ZT:time in LD, in which lights are turned on at ZT0 and off at ZT12) 11, 16 and 20, Per runs at 9.1 s on a sucrose gradient, between the aldolase (7.8 S; relative molecular mass 158,000 (M_r 158K)) and catalase (11.3 S, 232K) markers. This is considerably larger than the Per monomer (127K)^{22,23}, suggesting that Per is present in a protein complex. At ZT2 (in the morning), Per runs at 5.3 s, between the albumin (4.3 S, 67K) and aldolase (7.8 S, 158K) markers; this is close to Per's monomeric size. The analysis of more time points (data not shown) indicates that Per is mostly present in the large complex between ZT10 and ZT20, which is the accumulating phase of the Per cycle⁹; between ZT1 and ZT4 (the declining phase), Per is mostly in the small form. Between these two phases (from ZT22 to ZT0), the large and small forms are mixed. That Per complexes are temporally regulated suggests

FIG. 2 Per homodimers are a minor portion of Per complexes. Fly head extracts with single or double-tagged Per were immunoprecipitated (IP) with anti-HA (12CA5) or anti-myc (9E10) monoclonal antibodies. Top, western blots with anti-HA and anti-myc of the IPs with anti-HA. Bottom, western blots with anti-myc and anti-HA on the IPs with anti-myc. The following fly lines were used: *myc/HA*, double-tagged line *per⁰¹;per-HA/per-HA;per-myc/per-myc*. HA, *per⁰¹;per-HA/per-HA;ry⁵⁰⁶*. *myc*, *per⁰¹;per-myc/per-myc*. *myc* + HA denotes mixed extracts of *myc* and HA lines. If the complex contains predominantly homodimers, in *myc/HA* flies the amount of Per-myc co-immunoprecipitated by anti-HA should be half the amount of Per-HA in the same IP. But this was not observed. Homodimers were seen in all three time points of *myc/HA*, and all were only 6% by a BioRad Molecular Imager quantification. The Per bands seen in the HA and *myc* + HA lanes of the bottom right panel were due to a tiny contamination of anti-HA with anti-myc in IP, and were subtracted from the quantification of Per in *myc/HA*. Bands not marked are nonspecific.

METHODS. Anti-HA (15 μ g) or anti-myc antibody was preincubated with 30- μ l 1:1 slurry of GammaBind Plus Sepharose beads (Pharmacia) in 1 ml extraction buffer for 1 h at 4 °C with rotation. After washing, beads were incubated with 400- μ l fly head extracts for 4 h at 4 °C with rotation. Three 10-min washes were followed by boiling beads in SDS sample buffer. Samples were fractionated by 6% (29:6:0.4) SDS-PAGE, and western blotted with anti-HA or anti-myc. Efficiencies of IPs with N- or C-terminally tagged lines were always greater than 80% (see Fig. 4a), indicating that the tags were not masked in protein complexes, and did not interfere with each other.



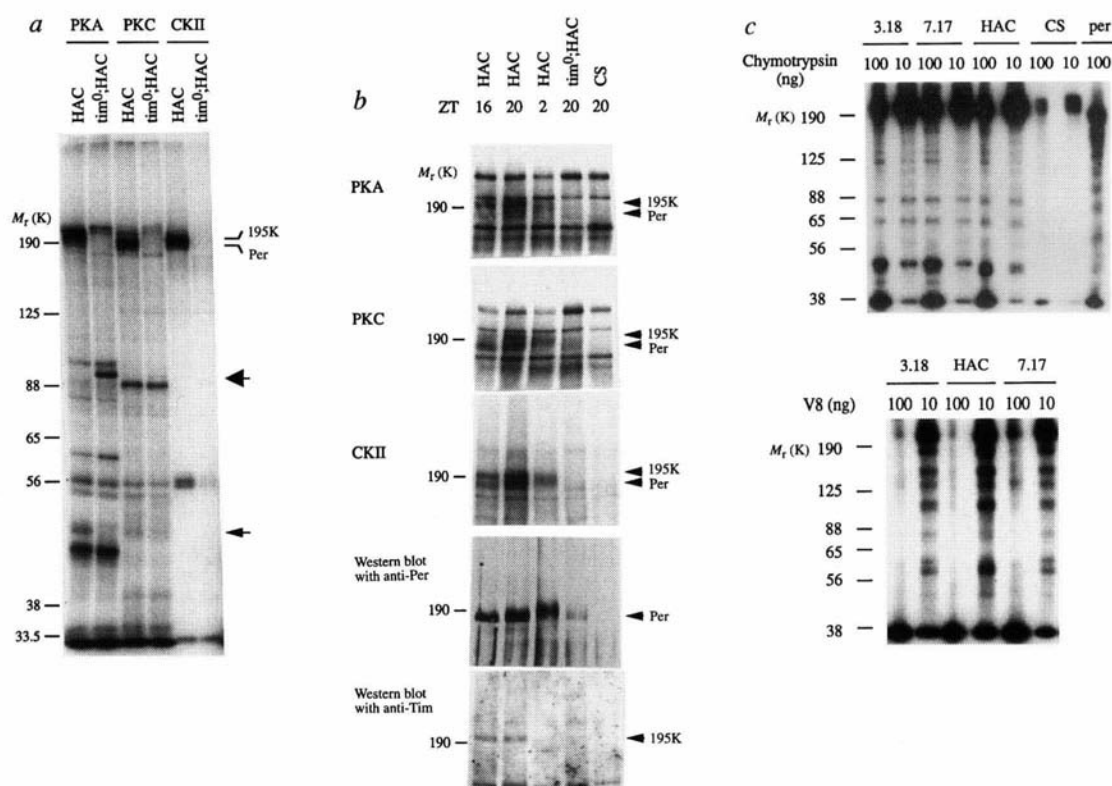


FIG. 3 The 195K protein co-immunoprecipitated with Per is Tim. Proteins immunoprecipitated with anti-HA were labelled by phosphorylation with three commercial kinases: PKA catalytic subunit, PKC, or CKII. We used *tim*⁰; HAC and Canton-S (CS, non-tagged wild-type) flies as negative controls. **a**, Labelled proteins from HAC and *tim*⁰:HAC, both from ZT20, on 7.5% SDS-PAGE. The broad band near 190K actually contains two bands, Per and a 195K protein (see **b**). Small arrow points to a unique, non-reproducible band. Large arrow points to a band only in the negative controls CS and *tim*⁰:HAC. **b**, Fractionation by 6% (29.6:0.4) SDS-PAGE. All three kinases revealed only two distinct bands that were absent in CS lanes (arrowheads). **c**, Partial proteolysis of the 195K protein and *in vitro* expressed Tim. The lanes 3.18 and 7.17 are two independent full-length *tim*-HA constructs for *in vitro* translation; HAC, the 195K protein purified from HAC; CS, the gel slice equivalent to 195K from CS as a negative

control; *per*, *in vitro* translated PER also as a negative control. **METHODS.** Fly head extracts (400 μ l) were immunoprecipitated with anti-HA. After 3 washes, the beads were incubated with 100 μ l kinasing buffer, 0.5 μ l [γ -³²P]-ATP (6,000 Ci mmol⁻¹, 150 mCi ml⁻¹) and 1 mU PKA, 0.04 mU PKC or 0.1 mU CKII (all from Boehringer Mannheim) for 30 min at 30 °C (PKA and PKC) or 37 °C (CKII). Reactions were stopped with 10 mM EDTA and beads washed and boiled in SDS sample buffer. The anti-Tim polyclonal antibody was generated in mice with a GST-Time(505–906) fusion protein. To further clean up the IPs for partial proteolysis, 200 μ l of fly head extract was immunoprecipitated with anti-HA, eluted with HA peptide, and the eluate was immunoprecipitated with anti-Per, followed by PKA labelling. *In vitro* translated proteins (50 μ l) (following Promega instructions) were diluted with 1 ml extraction buffer, immunoprecipitated with anti-HA, and labelled by PKA. In-gel partial proteolysis was performed as described²⁷.

that they are of biological significance, and not due solely to protein aggregation.

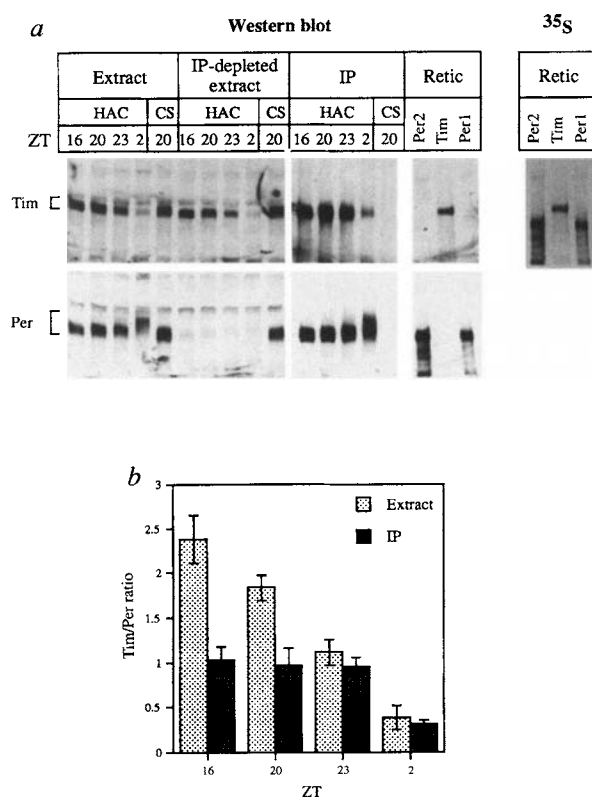
To examine further the relevance of the large complex, we analysed other genotypes (Fig. 1b). In the arrhythmic *hspcper-23a* transgenic line⁵, in which Per is dramatically overexpressed from a heat-shock promoter, the protein is in the low-M_r form. Per is also mainly in this form in the *rh-per* line¹², which expresses high levels of Per that extinguish circadian cycling in photoreceptor cells. As these constructs express more Per than wild-type flies, the large complex is unlikely to be due to aggregation. Finally, no large complex was observed in extracts from *tim*⁰, the arrhythmic *timeless* null mutant^{16,20}. Thus rhythmicity and the large complex are correlated, and its absence in *tim*⁰ flies may be related to the potent post-transcriptional effect of Tim on Per^{13,15}.

Tim is in the large Per-containing complex

The complex was also analysed by gel filtration (Fig. 1 legend). Taken together with the sucrose-gradient data, its estimated size (M_r 337K) indicates that it could be due to Per homodimers; indeed, Per fragments are able to homodimerize through their PAS domains, *in vitro* and in yeast^{24,25}. To test for Per homodimers, we used two biologically active epitope-tagged Per transgenes²⁶ to create a double-tagged homozygous rhythmic line (*per*⁰¹; *per*-HA/*per*-HA; *per*-myc/*per*-myc). If the large complexes contain predominantly Per homodimers, then anti-haemagglutinin (HA) anti-

body should co-immunoprecipitate considerable quantities of Per-myc at ZT16 or ZT20 but not at ZT2. However, there is less than one-tenth as much Per-myc as Per-HA, and this does not vary as a function of circadian time (Fig. 2, top). Similar results were obtained when Per-HA was co-immunoprecipitated with anti-myc antibody (Fig. 2, bottom), indicating that Per homodimers account for no more than a minor fraction of the Per-containing complex.

To visualize the predicted Per-associated proteins, we labelled the proteins on beads with commercial kinases and [γ -³²P] ATP after immunoprecipitation of Per-HA-containing extracts with anti-HA antibody. With all three kinases, only one extra band was detected (Fig. 3a). As the apparent M_r of this additional protein (195K) is slightly higher than that of Per, a doublet was visible only on low-percentage acrylamide gels (Fig. 3b). Western blots show that the lower band is indeed Per (Fig. 3b). The two proteins were labelled differentially: protein kinase A (PKA) preferentially labelled the upper band, protein kinase C (PKC) the lower band, and casein kinase II (CKII) labelled both bands equally (Fig. 3b). Consistent with previous results (Fig. 1 and ref. 15), the novel upper band was not present in immunoprecipitates from ZT2 flies, and only a faint Per band was detected from *tim*⁰; *per*-HA flies. The doublet bands were the only proteins dependent on the presence of the HA tag, that is, that were clearly missing in immunoprecipitates of Canton-S (not-tagged) extracts.



During the course of these studies the *tim* gene was cloned and sequenced¹⁶. The size of the predicted Tim is somewhat greater than that of Per, and the absence of the 195K upper band in *tim*⁰ extracts also suggested that it might be Tim. Indeed, this band was detected with an anti-Tim polyclonal antibody in the per-HA immunoprecipitates of ZT16 and ZT20 but not ZT2 nor from *tim*⁰, per-HA or CS extracts (Fig. 3b, bottom). Because there was some residual signal in the doublet region of the CS extract-derived lane, and to verify that Tim was the only protein in the 195K band, we compared the partial proteolysis patterns²⁷ of this protein with full-length Tim, expressed in a reticulocyte lysate system (Fig. 3c). Consistent with expectation, Tim has an apparent M_r of 195K on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). (The theoretical M_r of Tim is 156K (ref. 16), but like Per it migrates aberrantly on SDS–PAGE; Per has an apparent M_r of 185K, rather than the predicted 127K (ref. 9).) For both *Staphylococcus aureus* V8 protease and chymotrypsin, the patterns are virtually identical between 195 K protein and Tim, except that a few bands are slightly shifted, possibly owing to differential phosphorylation or to the presence of the HA tag in the *in vitro* translated Tim (Fig. 3c). We conclude that the 195K protein is indeed the *timeless* gene product.

Per and Tim are heterodimeric partners

The size of the large Per-containing complex is consistent with the predicted size of a Per–Tim heterodimer. To verify that Per and Tim are stoichiometric partners, we calibrated our anti-Tim and anti-Per antibodies to estimate the relative levels of the two proteins in HA-immunoprecipitates as well as in extracts. *In vitro* translated Tim and Per were used as standards, and were also labelled with [³⁵S]-Met to measure their relative amounts. Fly head extracts, immunodepleted extracts and immunoprecipitates of different time points, and *in vitro* translated proteins were electrophoresed and sequentially assayed, first with anti-Tim and then anti-Per antibodies (Fig. 4a). The quantification (Fig. 4b) indicates that Per and Tim are present in equal amounts in the immunoprecipitates at ZT16, ZT20 and ZT23, consistent with a 1:1 stoichiometric relationship of the two proteins. At ZT2, the immunoprecipitates contain much less Tim than Per, consistent

FIG. 4 Stoichiometric characterization of Per and Tim. The relative amounts of *in vitro* translated Per and Tim were measured from ³⁵S labelling and were used to calibrate the anti-Per and anti-Tim antibodies. The antibodies were then used to estimate the relative levels of Per and Tim in HA-immunoprecipitates (IP) and in extracts. a, Western blots with anti-Tim and anti-Per, and autoradiogram of ³⁵S-labelled proteins. HAC, per-HAC flies; CS, non-tagged wild type as control; Retic, *in vitro* translated proteins from reticulocyte lysate. Twice as much Per was loaded in Per2 as in Per1. b, Quantitative ratios of Tim/Per in extracts and IPs of various time points, plotted as mean $\pm$ s.e.m. ($n = 4-6$).

METHODS. Tim and Per were *in vitro* translated in the presence of [³⁵S]-Met. To load similar amounts of Per and Tim, 2 μ l (Per2) or 1 μ l (Per1) of Per-containing retic lysate and Tim from 12.5 μ l retic lysate (concentrated by immunoprecipitation with anti-HA) were loaded on 6% (29.6:0.4) SDS–PAGE. Extracts (10 μ l), immunodepleted extracts (10 μ l), and immunoprecipitates (from 100 μ l of extracts) of various time points were also loaded. The blot was incubated with anti-Tim and quantified in a BioRad Molecular Imager for chemiluminescence. It was then blotted with anti-Per and quantified. Undesired stripping, which may cause loss of signal, was avoided owing to the difference in secondary antibodies (anti-mouse for anti-Tim, and anti-rat for anti-Per). After rinsing off ECL reagents, the blot was again quantified in the Phosphorimager (BioRad) for ³⁵S. The relative affinity of anti-Tim and anti-Per antibodies was calculated based on the number of methionines (Per:Tim, 35:46). To show that the antibody recognition was not altered by phosphorylation, we treated both fly and *in vitro* translated proteins with phosphatase and found that the quantifications were not affected (data not shown).

with the observation that Per is mostly in monomer form at this time. The quantifications also indicate that the extracts contain about twice as much Tim as Per at ZT16 and ZT20 (Fig. 4b); this explains why approximately half of Tim remains in the depleted extracts, despite very efficient (> 80%) immunoprecipitation of Per and a 1:1 stoichiometric ratio (Fig. 4a, b). Thus, during much of the night, most Per is complexed with Tim but a substantial fraction of Tim is not complexed with Per ('free Tim'; free of Per, but possibly complexed to other proteins; H.Z. and M.R., unpublished data).

Taken together, the data indicate that Per and Tim are heterodimeric partners in flies. First, the large Per-containing complex is absent in the *tim*⁰ mutant. Second, we detect only one other major band in the complex, which reacts with an anti-Tim antibody and has a proteolytic pattern that matches that of Tim. Third, the estimated size of the complex (M_r 337K) is reasonable for the sum of the M_r of Per and Tim (127K + 156K = 283K). Fourth, Per homodimers account for no more than a small fraction of the complex, although they could be biologically relevant^{24,25}. Fifth, equal amounts of Tim and Per are co-immunoprecipitated; this ratio and the size of the complex are only compatible with a heterodimeric structure. However, we cannot exclude the presence of some mixed forms and additional components especially proteins that are of low M_r , substoichiometric, loosely associated, or that fail to label well with all three kinases. The absence of a PAS dimerization domain²⁴ from Tim¹⁶ indicates that the complex is the first example of an *in vivo* heterodimer between a PAS and a non-PAS protein; intramolecular interactions with the Per PAS domain might affect interactions between Tim and Per²⁵.

Tim cycling

Because there is hardly any large Per-containing complex in the early morning, we considered that Tim cycling might be relevant to the temporal disappearance of Per–Tim heterodimers. To investigate this possibility, we initially examined the circadian pattern of Tim expression (Fig. 5a). Tim levels cycle in constant dark (DD) as well as LD and they peak at about ZT19. The pattern is similar to that of Per¹² (Fig. 5a) and is reminiscent of *tim* mRNA undergoing circadian oscillations in both LD and DD with

amplitudes and phases similar to those of *per* mRNA¹⁷. Tim also undergoes a temporal change in mobility, although less dramatic than that observed with Per⁹: the Tim band becomes broader and decreases in mobility as a function of time (Fig. 5a). Phosphatase treatment indicates that a substantial fraction of the mobility

change is due to phosphorylation, as previously shown for Per⁹ (Fig. 5b).

Careful inspection of the Tim and Per cycling curves (Fig. 5a and data not shown) indicates a quantitative difference: during the late night/early morning, the decrease in Tim levels is rapid and

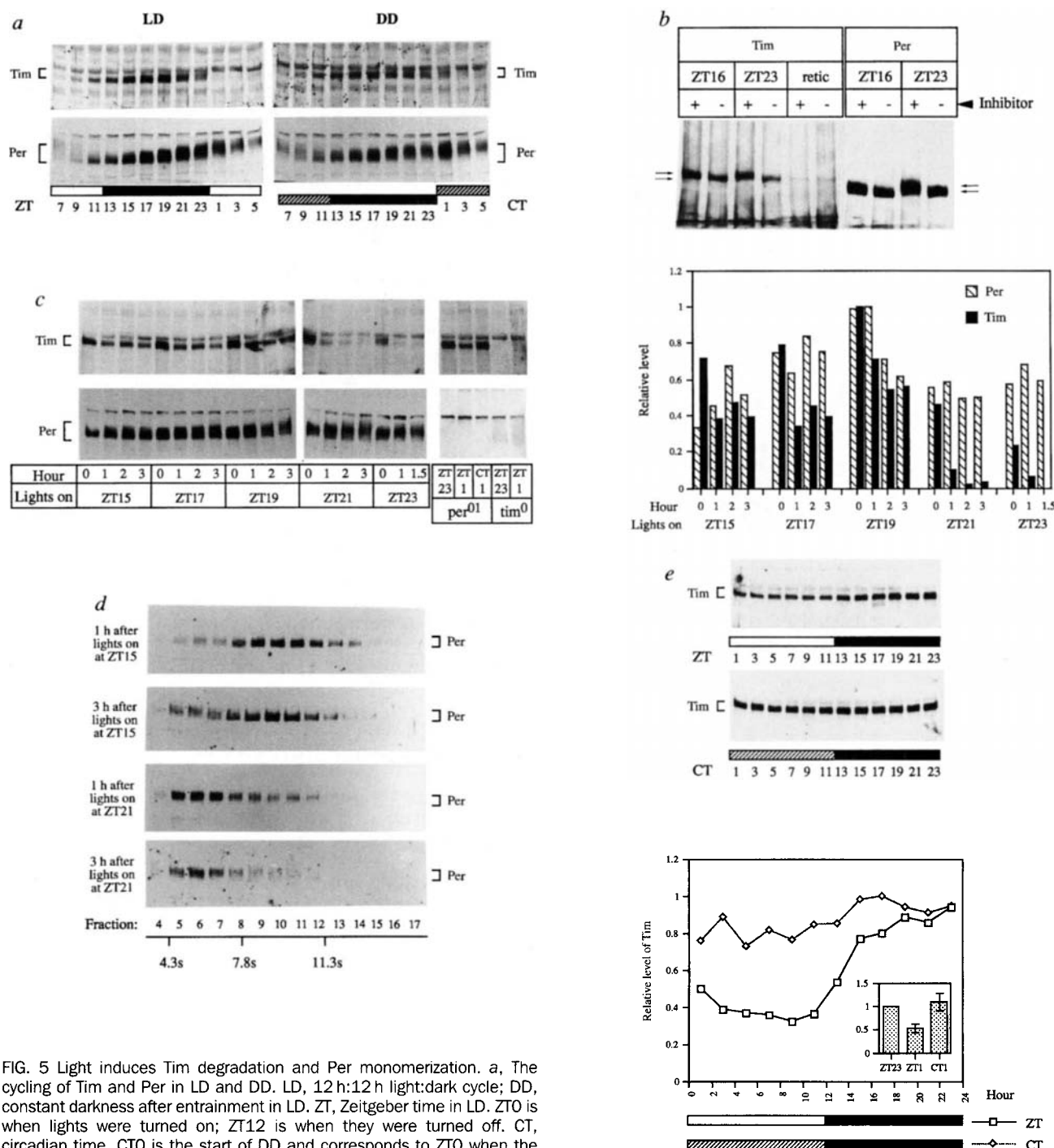


FIG. 5 Light induces Tim degradation and Per monomerization. **a**, The cycling of Tim and Per in LD and DD. LD, 12 h:12 h light:dark cycle; DD, constant darkness after entrainment in LD. ZT, Zeitgeber time in LD. ZTO is when lights were turned on; ZT12 is when they were turned off. CT, circadian time. CT0 is the start of DD and corresponds to ZT0 when the lights do not turn on, that is, CT1 is following ZT23, and CT23 is 22 h later than CT1. Open bar, day; solid bar, night; hatched bar, subjective day. **b**, Phosphatase treatment of Tim and Per. Immunoprecipitated Per-Tim complexes were treated with potato acid phosphatase (PAP) on beads in the presence (+) or absence (-) of a phosphatase inhibitor, 100 mM Na₃PO₄. Double arrows indicate initial and dephosphorylated forms of Tim (left) and Per (right); retic, *in vitro* translated Tim (Tim-HA) had no mobility change. **c**, Premature light during the night induces degradation of Tim but not Per. Bottom, quantification of the top. Lights on, the time when lights were turned on. Hour, hours after lights on. Hour 0 is immediately before lights were turned on. As a comparison, time points of *per*⁰¹ and *tim*⁰ around normal lights on at ZT0 are also shown. **d**, Sucrose gradients of Per after

lights on. **e**, Tim levels in *per*⁰¹ flies in LD and DD. Bottom, the quantification of the top. The inset compares Tim levels 1 h after lights on (ZT1) with lights not on (CT1), plotted as mean $\pm$ s.e.m. ($n = 3$).

METHODS. For **a**, **c** and **e**, 10 μ l extract (50 μ g total protein) was loaded on each lane for western blotting. Nonspecific cross-reacting bands serve as internal controls for equal loading. ECL was quantified in a BioRad Molecular Imager. For **b**, PAP treatment was performed as described⁹ with the following modification: 0.01–0.1 U of PAP (Boehringer Mannheim) was used for each immunoprecipitation from 100 μ l of extract. For **a**–**c**, the same blots were probed with anti-Tim and then with anti-Per. For **d**, the protocol in Fig. 1 was followed.

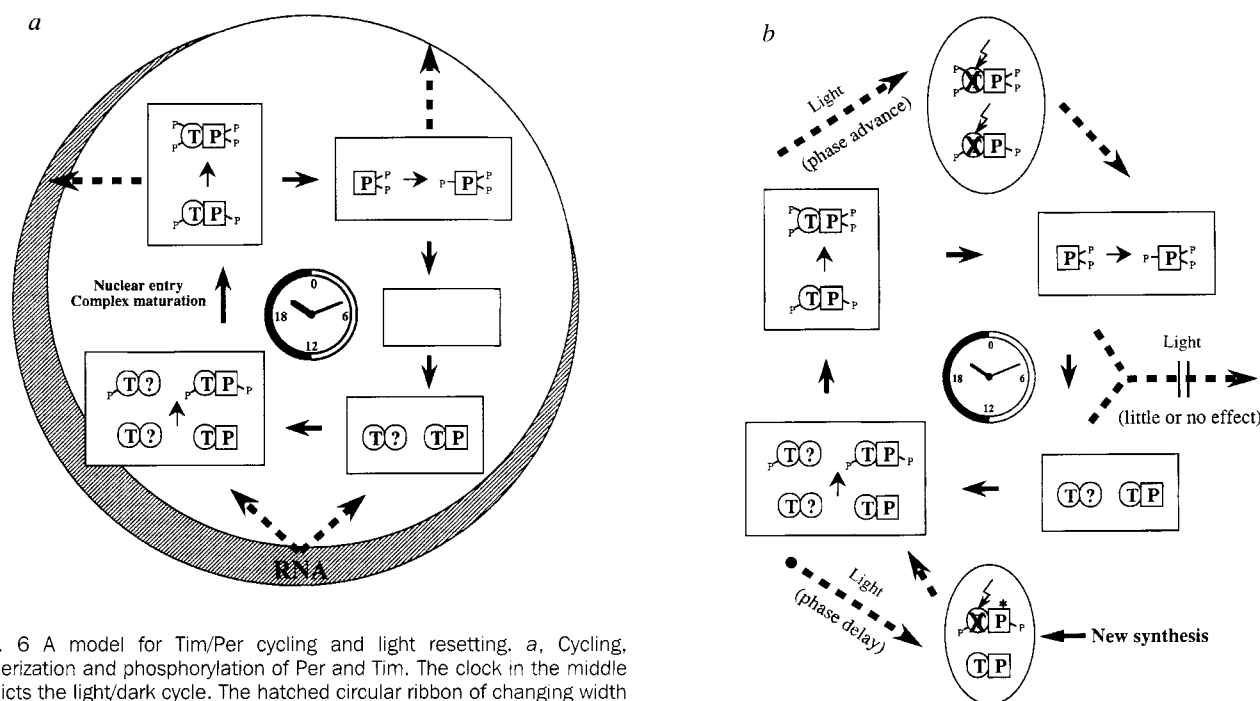


FIG. 6 A model for Tim/Per cycling and light resetting. *a*, Cycling, dimerization and phosphorylation of Per and Tim. The clock in the middle depicts the light/dark cycle. The hatched circular ribbon of changing width illustrates RNA cycling of both *per* and *tim*, which peaks at ~ZT15. Circadian cycling of the levels, dimerization and phosphorylation of Per and Tim is represented in five typical stages. **T**, Tim; **P**, Per; **p**, phosphorylation on proteins. The 'free' Tim, which does not associate with Per, is depicted as interacting with an unknown factor (?) which could be Tim itself; Tim is in large complexes even in the absence of Per (H.Z. and M.R., unpublished data). Per and Tim are believed to participate in the feedback regulation of their own transcription^{7,17} (broken arrows at top). However, which of the many forms of Per and/or Tim are the active factors is as yet unclear. *b*, Light resets the clock by changing the states of Tim and Per. One of the clock's initial responses to light is the degradation of Tim. During much of the subjective day, the clock is relatively insensitive to light, as there is little Tim. During the early night, light causes phase delays as Tim

degradation sets the protein states of both Per and Tim back to an earlier point (the oval at the bottom). The released Per (marked by an asterisk) could become associated with the undegraded 'free' Tim. New synthesis (also broken arrows at bottom in Fig. 6a) refers to input to the protein pool when *tim* and *per* RNA levels are substantial. During the late night, light causes phase advances because the degradation of most of Tim releases highly phosphorylated nuclear Per and thus sets the clock to a later point (the oval at the top). The transition from phase-delay zone to phase-advance zone occurs around CT18, as a result of the progressive phosphorylation and/or nuclear entry.

complete in LD, and precedes the more gradual decrease that Per undergoes (Fig. 5a, compare Tim ZT23, ZT1 with Per ZT23, ZT1; also see Fig. 4b). This suggests that the conversion of Per from dimer to monomer may be caused by the disappearance of Tim. Moreover, the morning decline in Tim levels is more rapid in LD than in DD (Fig. 5a; compare Tim CT1, 3 with Tim ZT1, 3). As there are very low *per* and *tim* mRNA levels at these cycle times^{7,17}, Tim disappearance is almost certainly due to Tim turnover, and this is probably facilitated by the lights being turned on at ZT0.

Light causes Tim degradation

As light is the major temporal cue that entrains and phase-shifts circadian rhythms, we considered that it might act more generally to cause Tim degradation. To test this possibility, we exposed flies to light at different times of night and examined the levels of both Tim and Per by western blotting (Fig. 5c). At all times, Tim levels decreased in less than 1 h of light, and there was no further decrease after 2 or 3 h. (The decrease was significant after only 20 min of light or short light pulses; H.Z. and M.R., unpublished data.) Although less Tim was degraded in the early evening than later at night, in all cases only the higher-mobility (hypophosphorylated) forms of Tim persisted. They probably reflect newly synthesized Tim that has not yet undergone substantial phosphorylation. In addition, the lower mobility (more phosphorylated) forms of Tim, which predominate late at night when the fraction of degraded Tim is high, may be preferential degradation substrates. Consistent with the quantitative differences between Tim degradation at ZT15 and ZT21, Per's shift from dimer to monomer is virtually complete 1 h after lights were turned on at ZT21, but barely apparent at ZT15 (Fig. 5d). The same light protocol affects

Per levels to a much lesser extent (Fig. 5c); they were not very different from what would normally be observed at these times. However, Per is noticeably more phosphorylated after exposure to light at certain times (for example, Fig. 5c, ZT19, 21), which probably reflects further phosphorylation after conversion to the monomer form. Although the response of Per suggests that it is affected by light less directly than Tim, the *per* mutants are known to affect the magnitude of the light response²⁸. A parsimonious explanation is that they act by affecting the structure of the Per–Tim heterodimer, which is a major light target.

In *tim*⁰ flies, there is substantial *per* mRNA but little Per, suggesting that Per is unstable in the absence of Tim¹⁵. To examine the reciprocal dependence of Tim levels on Per, we assayed Tim in *per*⁰¹ flies. Surprisingly, Tim levels are quite high in the absence of Per (Fig. 5e): the night-time level is at least half of the peak level observed in wild-type flies (data not shown; also see Fig. 5c). Remarkably, there is an oscillation of Tim levels in LD but not in DD (Fig. 5e, bottom). Tim levels are relatively low during the light phase and increase after lights are turned off at ZT12. The light-induced decrease is very rapid (comparing ZT23, 1 and CT1; Fig. 5e inset; also see Fig. 5c). Because *tim* RNA levels do not oscillate in *per*⁰¹ (ref. 17), the effect of light on Tim must be mediated by a post-transcriptional mechanism, for example, degradation or translational inhibition. A light-induced decrease in Tim levels is also evident in wild-type flies at ZT21–ZT24 when *tim* RNA levels are extremely low¹⁷, so a translational mechanism is very unlikely. The *per*⁰¹ results demonstrate that light-induced Tim degradation is Per and clock independent.

Regulation of the Per–Tim cycle

Light resetting takes place rapidly in *Drosophila* (within 1–3 h)²⁹.

Thus it is likely that light induces a rapid change in the level or activity of one or a few clock components, so that the clock is shifted to a new phase. In *Neurospora*, it has been proposed that *frq* transcription is the relevant light-sensitive clock component³⁰. In contrast, we have not observed strong and rapid effects of light on *per* RNA levels (M.R. *et al.*, manuscript in preparation). We therefore suggest that transcription is affected less directly, and that Tim and/or the Tim-Per heterodimer are more direct light-sensitive targets of the *Drosophila* clock.

Our observations indicate that light and the post-transcriptional dynamics of the Per-Tim cycle are important for circadian time-keeping (Fig. 6a). As Per is unstable in the absence of Tim, but Tim is quite stable without Per, Tim must make a major post-transcriptional contribution to accumulation of Per during the early evening. In the late night and early morning, Tim degradation gives rise to Per monomers, which then decay during the morning. Because both Per and Tim participate in transcriptional feedback regulation of both genes^{7,17}, the definition of the Per-Tim heterodimer also explains this reciprocal autoregulation. It is not yet known whether the heterodimer participates directly in transcriptional regulation. Temporal phosphorylation of the complex (Per and Tim) may contribute to features of transcriptional regulation (for example, the timing of nuclear entry) or to gating of the protein cycle (for example, light-induced degradation of Tim). Complex dissociation mediated by signal-dependent destabilization of one subunit is not without precedent, for example NF- κ B/I κ B³¹, where I κ B degradation activates NF- κ B or cyclin/cdk^{32,33}, where cyclin degradation inactivates cdk. In contrast, the biochemical mechanism of light-induced Tim degradation is

completely unknown. Light might cause a modification of Tim, or it might lead to the activation of a dedicated protease.

The rapid effect of light on Tim can also account for entrainment to 24-h light cycles. Light advances the cycle at ZT0 by causing degradation of Tim. It also delays the cycle until ZT12, when light inhibition of Tim-Per accumulation is relieved by the light-dark transition (M.R. *et al.*, manuscript in preparation). It can also account for phase-shifting light pulses having three different time-dependent effects: delays in the early evening; advances late at night; and relatively little effect during much of the subjective day²⁸ (Fig. 6b). (1) In the early evening, degradation of Tim is rapid but modest. This is probably due to there being substantial mRNA levels at these times, so the degraded Tim can be replaced by newly synthesized Tim, and the system returns to its pre-light position after a phase delay. Also relevant may be the phosphorylation states of the Tim-Per complex: hypophosphorylated Tim may be a relatively poor substrate for the degradation response. (2) Late at night, most of Tim is degraded in response to light and most of Per is released, because there is little or no *tim* mRNA for replacement. The disappearance of the mature complex and/or release of mature Per occurs prematurely and phase-advances the clock. (3) During much of the subjective day, there is little Tim and therefore little or no light response. We cannot exclude the possibility that other components of the system undergo circadian changes that also influence the magnitude and direction of the light response. However, the effects of light on the degradation of Tim can explain the major features of entrainment and phase resetting, suggesting that it may be close to the initial response of the circadian oscillator to light. □

Received 15 January; accepted 23 February 1996.

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ACKNOWLEDGEMENTS. M.P.M. thanks M. W. Young for support. We thank I. Ederly for originating biochemical studies on Per; M. E. Dembinska for generating anti-Per antibodies; U. Schibler for the mouse-antibody protocol; J. C. Hall and J. E. Rutla for discussion; M. W. Young, H. V. Colot, J. E. Rutla and R. Allada for comments on the manuscript; E. Dougherty for photographic assistance; and L. A. Monaghan for secretarial assistance. We appreciate communication of related unpublished observations from M. W. Young. Z.Q. is a Howard Hughes Medical Institute fellow of the Life Sciences Research Foundation.

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Substantial outgassing of CO from comet Hale-Bopp at large heliocentric distance

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WHEN comet C/1995 O1 (Hale-Bopp) was discovered¹, at a distance of seven astronomical units from the Sun, it was more than one hundred times brighter than comet Halley at the same distance. A comet's brightness is derived from the reflection of sunlight from dust grains driven away from the nucleus by the sublimation of volatile ices. Near the Sun, sublimation of water ice (a main constituent of comet nuclei) is the source of cometary activity; but at its current heliocentric distance, Hale-Bopp is too cold for this process to operate. Other comets have shown activity at large distances², and in the case of comet Schwassmann-Wachmann 1, carbon monoxide has been detected in quantities sufficient to generate its observed coma^{3,4}. Here we report the detection of CO emission from Hale-Bopp, at levels indicating a very large rate of outgassing. Several other volatile species were searched for, but not detected. Sublimation of CO therefore appears to be responsible for the present activity of this comet, and we anticipate that future observations will reveal the onset of sublimation of other volatile species as the comet continues its present journey towards the Sun.

The observations were conducted with the Institut de Radio Astronomie Millimétrique (IRAM 30-m radio telescope⁵ at Pico Veleta, Spain). We observed several lines simultaneously by using three receivers from a choice of four, namely one 3-mm, one 2-mm and two 1.3-mm receivers. Two observing modes were used in turn: frequency switching, or rapid position switching by tilting the secondary mirror. At 230 GHz, which is the frequency of the CO $J(2-1)$ rotational line, the telescope beam width at half-power is 10.4 arcsec and the main beam efficiency is 0.39. Although the comet was close to the Galactic plane, the comet spectrum was contaminated by Galactic CO emission in only one of our observing periods.

We searched for the CO $J(2-1)$ line as early as 16 and 23 August 1995, but found only a broad feature which may be spurious (see Table 1). It was clearly detected on 20–21 September⁶ (in agreement with other observations conducted independently at the James Clerk Maxwell Telescope at Hawaii⁷) and observed at several subsequent periods. The same CO line was also independently observed in December at the National Radio Astronomical Observatory 12-m telescope at Kitt Peak⁸. The log of our observations is given in Table 1 and the spectra are shown in Fig. 1. The CO $J(1-0)$ line at 115 GHz was only marginally detected, with an area (intensity integrated over the line) of 0.04 ± 0.01 K km s⁻¹, when averaging all our observations.

The CO line is resolved. It is consistently centred on -0.38 ± 0.07 km s⁻¹ and its width at half-maximum ranges from 0.23 to 0.40 km s⁻¹. The line profile is a combination of the thermal motion and of the outflow velocity of the cometary atmosphere. The gravitational attraction and the rotation of the nucleus have negligible effects on the velocity field. The offset in velocity shows that the atmosphere is mainly expanding towards the observer, that is, also towards the Sun, as expected for sublimation occurring on the sunlit side of the nucleus. The same effect was observed in

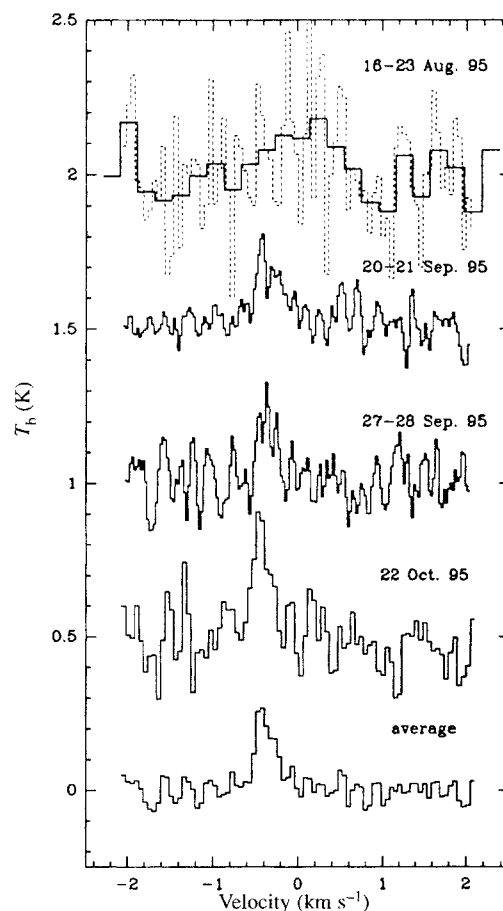


FIG. 1 The $J(2-1)$ line of carbon monoxide in comet Hale-Bopp, observed at the IRAM 30-m telescope at different times. The spectrum obtained on 16–23 August is shown with full spectral resolution (dotted line) and after smoothing (solid line). The bottom spectrum is the average of the September and October observations. The spectra were analysed with an autocorrelator with a resolution set to 20 or 40 kHz. The latter corresponds to a Doppler velocity resolution of 0.05 km s⁻¹. The intensity scale is brightness temperature.

the CO lines of comet Schwassmann-Wachmann 1 (refs 3, 4) and in the molecular radio lines of several other comets. The line is narrower than the molecular radio lines observed in comets at about 1 AU from the Sun^{9,10} which have typical widths of 1 km s⁻¹ or more. It is, however, wider than the extremely narrow CO line (0.15 km s⁻¹) observed⁴ in Schwassmann-Wachmann 1. These line shapes reflect the kinematics of the gas jets in the coma of comet Hale-Bopp, which are the counterparts of the dust jets seen in the visible images of this comet¹¹.

The CO line intensities have been converted into CO production rates (Table 1) using a simplistic model¹² which assumes an isotropic expansion of the atmosphere with a velocity of 0.5 km s⁻¹ and a CO rotational temperature of 10 K. This temperature, which is in agreement with our marginal detection of the CO $J(1-0)$ line, is that retrieved from our previous observations of the CO $J(2-1)$ and $J(1-0)$ lines in comet Schwassmann-Wachmann 1 at IRAM⁴. Such a low temperature is predicted by models of the cometary atmosphere as due to adiabatic expansion. The CO rotational temperature is unlikely to be constant in the coma, but its value is not critical: an excursion in the 6–30 K range does not change the production rate by more than a factor of two. The lifetime of the CO molecule—of the order of one year at 6.5 AU—is not a crucial parameter because the scale-length of the molecule is much larger than the radio telescope field of view. Isotropy of the coma is

TABLE 1 CO J(2–1) observations

Date (1995)	r_h (AU)	Δ (AU)	$\int T_b dv$ (K km s ⁻¹)	$\langle v \rangle$ (km s ⁻¹)	FWHM (km s ⁻¹)	$Q[CO]$ (10 ²⁸ s ⁻¹)
16, 23 Aug.	6.9	6.3	0.190 ± 0.040*	0.00 ± 0.08	0.77 ± 0.17	
20–21 Sept.	6.7	6.5	0.090 ± 0.007	–0.33 ± 0.02	0.40 ± 0.04	2.0 ± 0.2
27–28 Sept.	6.6	6.5	0.060 ± 0.009	–0.36 ± 0.02	0.23 ± 0.04	1.3 ± 0.2
22 Oct.	6.4	6.7	0.120 ± 0.018	–0.43 ± 0.02	0.26 ± 0.05	2.6 ± 0.4

Abbreviations used: r_h , Sun–comet distance; Δ , Earth–comet distance; $T_b dv$, line area (mean brightness temperature integrated over the line); $\langle v \rangle$, line centre; FWHM, full-width at half-maximum of the line; (these three last quantities being derived from gaussian fits); $Q[CO]$, carbon monoxide production rate in molecules s⁻¹.

* The August spectrum, of poor quality, shows a feature which is broader and offset in velocity with respect to the lines observed in September and October; it may be spurious; when integrated over a [–0.7, 0.0] km s⁻¹ window, the line area is 0.061 ± 0.038 K km s⁻¹.

contradicted by the observed line shape. We are at present investigating asymmetric models. The observed line profiles are well represented by a gas output collimated in a cone directed towards the observer with an aperture in the range 90–180° (full hemisphere). The calculated CO production rates are identical to those reported in Table 1 for the full-hemisphere assumption, and 40% lower for the 90° cone.

The data in Table 1 suggest that the CO production rate may be evolving with time. The comet activity is indeed varying, as is shown by the complex evolution of the dust jets.¹¹ Our observations are too sparse to investigate a possible relation of CO variability with the outbursts and jet evolution observed in the visible region. There is no progressive decrease of the outgassing, which supports the idea that the comet's exceptional brightness was not due to an outburst at the moment of (or just before) its discovery.

The CO production rate of comet Hale–Bopp (Table 1) is of the order of 2×10^{28} molecules s⁻¹, which is somewhat smaller than the CO production of comet Schwassmann–Wachmann 1 ($\sim 5 \times 10^{28}$ molecules s⁻¹; refs 3, 4). This rate (corresponding to $\sim 10^3$ kg s⁻¹) is enormous: it is as large as the CO production rate of comet Halley close to its perihelion. The total visual magnitude of comet Schwassmann–Wachmann 1 varies from ~ 18 to ~ 13.5 when undergoing an outburst. This brightness is due to the release of dust, but the derivation of dust production rates is highly dependent upon ill-known dust parameters such as density, size distribution, and velocity. Production rates ranging from 10 to 900 kg s⁻¹ have been evaluated^{13,14} for comet Schwassmann–Wachmann 1. Hale–Bopp being intrinsically brighter than Schwassmann–Wachmann 1 in outburst by at least a factor of 15, its dust-to-gas ratio is thus much larger (by a factor of 40 or more). Could other volatile species contribute to driving its dust coma?

Several other molecular species were unsuccessfully searched for at IRAM on 27–28 September and 22 October: hydrogen cyanide (HCN), formaldehyde (H₂CO) and methanol (CH₃OH). These molecules are known to exist in cometary nuclei, and have already been observed at radio wavelengths in several comets at smaller heliocentric distances^{10,15–17}. Their sublimation tempera-

tures are between those of carbon monoxide (24 K in cometary conditions) and water (150 K). They are therefore potential contributors to the distant activity of comet Hale–Bopp. The upper limits of these observations are reported in Table 2. They are converted into production-rate limits using the same models as those used in other comets for HCN (ref. 12), CH₃OH (ref. 16) and H₂CO (ref. 15), assuming here a coma temperature of 10 K. In August, the OH radical could not be detected by its 18-cm radio lines, searched for at the Nançay radio telescope (see Table 2), nor by its ultraviolet bands of its A–X system, searched for by the International Ultraviolet Explorer¹⁸. The corresponding upper limits on the water production rate are 2×10^{29} and 10^{29} molecules s⁻¹, respectively. In August, the detection of the violet system band of CN in the comet was announced¹⁹, with an intensity corresponding to $Q[CN] = 6 \times 10^{25}$ molecules s⁻¹. An observation of the ultraviolet spectrum of comet Hale–Bopp by the Hubble Space Telescope four days after our 22 October observation did not reveal the spectral signature of any atomic or molecular species (H. A. Weaver, personal communication).

The limits on OH radical production rate are ten times larger than that of CO. This is not a stringent constraint on the water outgassing: at 6.8 AU from the Sun, the nucleus temperature, in the fast-rotator approximation, is only 105 K, which is too low to permit significant water sublimation. The farthest distance at which OH (water) outgassing was ever recorded in a comet was 5.25 AU, in comet Bowell 1980 I (ref. 20); this outgassing was attributed to sublimation from superheated icy grains rather than from the nucleus. Such icy grains are probably also present in the coma of comet Hale–Bopp, as suggested by the observation of a shallow absorption feature at 2.04 μ m in the infrared spectrum of the comet²¹: one could expect their sublimation when the comet comes nearer to the Sun. The limits on the abundances, relative to CO, of HCN, CH₃OH and H₂CO are comparable to, or smaller than, those observed in other comets at ~ 1 AU from the Sun¹⁷. The CN production rate¹⁹ is smaller by a factor of two than the IRAM upper limit on HCN, therefore not in contradiction with CN coming from the photodissociation of HCN. We note that $Q[CN]/Q[CO]$ is 0.3%, which is about 20 times smaller than in typical comets observed around 1 AU. If one assumes that the

TABLE 2 Searches for other species

Date	Molecule	Line	Frequency (GHz)	3 σ upper limit		
				$\int T_b dv$ (K km s ⁻¹)	Q (10 ²⁶ s ⁻¹)	$Q/Q[CO]$
4–9 Aug.	OH*		1.67	<0.048*	<2,000.0	<10.0
27–28 Sept.	HCN†	J(1–0)	88.6	<0.032	<1.0	<0.8%
27–28 Sept.	H ₂ CO	2 ₁₂ –1 ₁₁	140.8	<0.029	<2	<1.5%
27–28 Sept., 22 Oct.	CH ₃ OH‡	(3, 0)–(2, 0)A ⁺	145.1	<0.037	<10.0	<8%

* Observed at the Nançay radio telescope with a beam 19' × 3.5' (right ascension × declination). For this entry, the line area is given in Jy km s⁻¹ per beam. The limit on the production rate is estimated using our radio OH model⁹.

† $Q[HCN]/Q[CO] = 0.3\%$ if all the observed CN (ref. 19) is coming from HCN.

‡ Several (3, K)–(2, K) lines were in the bandwidth of the spectrometer. The listed entry corresponds to the line expected to be the strongest.

abundance ratios are the same in comet Hale–Bopp as in other, less-distant, comets, this suggests that HCN, CH₃OH and H₂CO (which are highly polar species) are more easily retained in the matrix of water ice than carbon monoxide.

Comet Hale–Bopp will reach perihelion on 1 April 1997, at 0.91 AU from the Sun. We will have the possibility of monitoring its CO production rate, and of watching for the onset of sublimation of water and of other molecular species, as this productive comet approaches the Sun. The evolution of the relative abundances of the volatiles outgassed from the nucleus, owing to sublimation fractionation, could be studied. This would offer an opportunity to understand the mechanisms of cometary activity. □

Received 30 November 1995; accepted 7 February 1996.

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ACKNOWLEDGEMENTS. Scheduling of the observations reported here was possible thanks to IRAM staff and observers who accepted last-minute changes to their observing schedules. This work was partly supported by the Institut National des Sciences de l'Univers and the French Centre National de la Recherche Scientifique (CNRS). The Nançay Radio Observatory of the Observatoire de Paris is affiliated with the CNRS, and receives support from the Région centre. H.R. was supported by a fellowship from the European Space Agency.

Periodic collisions between the moon Prometheus and Saturn's F ring

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SATURN'S F ring, which lies 3,400 km beyond the edge of the main ring system, was discovered by the Pioneer 11 spacecraft¹ in 1979. It is a narrow, eccentric ring which shows an unusual 'braided' appearance in several Voyager 1 images² obtained in 1980, although it appears more regular in images from Voyager 2 obtained nine months later³. The discovery of the moons Pandora and Prometheus orbiting on either side of the ring provided a partial explanation for some of the observed features⁴. Recent observations of Prometheus^{5,6} by the Hubble Space Telescope show, surprisingly, that it is lagging behind its expected position by ~20°. By modelling the dynamical evolution of the entire Prometheus–F ring–Pandora system, we show here that Prometheus probably encountered the core of the F ring in 1994 and that it may still be entering parts of the ring once per orbit. Collisions with objects in the F ring provide a plausible explanation for the observed lag and imply that the mass of the F ring is probably less than 25% that of Prometheus.

Prometheus is known to produce density waves at resonant locations in Saturn's A ring and these lead to an exchange of angular momentum which should cause the orbit of Prometheus to expand. Thus a lag in the longitude of Prometheus is to be expected^{7–9}, but the observed value is at least an order of magnitude larger than that predicted. It has been suggested (P. D. Nicholson *et al.*, personal communication) that the excess lag may be the result of the passage of Prometheus through part of the F ring in the recent past. Here we show that it is likely that Prometheus is still colliding with F ring material.

Synnott *et al.*¹⁰ calculated orbital elements for the F ring and its satellites (Table 1). They were the first to note that, because the orbits of the F ring and Prometheus are eccentric and the maximum distance of Prometheus from Saturn (apoapse) lies close to the minimum distance of the F ring from Saturn (periapse), differential precession due to the oblateness of Saturn would result in close periodic approaches between Prometheus and the F ring. The closest distance, Δ , between the orbits depends on the orientation of the orbits and has a minimum value given by

$$\Delta_{\min} = a(1+e) - a'(1-e') \quad (1)$$

where a and e denote the semimajor axis and eccentricity of Prometheus with similar primed quantities for the F ring. From Table 1 we obtain $\Delta_{\min} \approx 100$ km. However, $\Delta = \Delta_{\min}$ only in the case when Prometheus is at its apoapse and the respective longitudes of periapse, $\bar{\omega}$ and $\bar{\omega}'$, are 180° apart. The oblateness of a planet causes $\bar{\omega}$ and $\bar{\omega}'$ to increase with time at different rates. By neglecting the mass of the F ring, Borderies *et al.*¹¹ showed that $\Delta_{\min}$ reduces to ~47 km every ~18 yr. As the longest radius of Prometheus is 70 km, Borderies *et al.*¹¹ pointed out that an actual collision between ring and satellite was possible.

We have developed a more complete model using secular perturbation theory. The dynamical evolution of the entire Prometheus–F ring–Pandora system can be studied by approximating each orbit to an elliptical wire of linear density $\rho = mr/2\pi ab$,

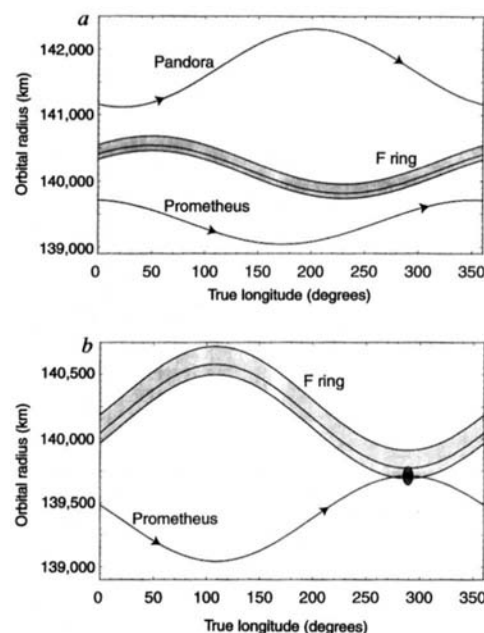


FIG. 1 a, Relative configurations of the orbits of Prometheus, the F ring and Pandora on 23 August 1981. The shaded area denotes the region enclosed by strands 80 km inside, and 140 km outside, the orbit of the F ring. Note that there is little likelihood of Pandora entering the F ring. b, Configurations for Prometheus and the F ring on 6 February 1994; the small oval represents Prometheus placed at the apoapse of its orbit (using the vertical scale). This plot was obtained by using the data in Table 1 to derive a secular solution to the gravitational interaction of both satellites and the ring. We have assumed uniform precession of all F ring components.

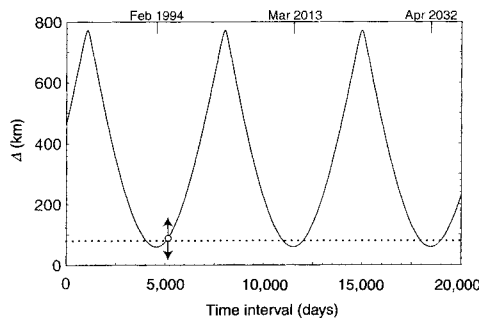


FIG. 2 The distance of closest approach, Δ , between the orbits of Prometheus and the main component of the F ring as a function of the number of days since 23 August 1981, showing minima in 1994, 2013 and 2032. The point denotes the value of Δ in mid-1995 and the vertical arrows denote the longest dimensions of Prometheus. The dotted line denotes the distance of the bright inner strand of the F ring. The plot is derived from the secular solution for the Prometheus-F ring-Pandora system for a zero-mass F ring.

where m is the mass of the object, r is its orbital radius and b is the semiminor axis of its orbit. The resulting secular interactions can be solved numerically for given starting conditions¹². Although the secular model breaks down when there is actual intersection of orbits, it has the advantage that it can include the effects of any number of orbiting, interacting masses. Figure 1a shows the orbital radii of the system as a function of true longitude using the values of a , e and $\bar{\omega}$ given in Table 1 for the epoch of 23 August 1981. We have solved the secular system to obtain an equivalent plot 4,550 d later on 6 February 1994 (Fig. 1b). Several Voyager 2 images show clear evidence of at least four separate strands which remain parallel over a range of $\sim 45^\circ$ in longitude^{13,14}. The brightest outer and inner strands are ~ 140 km and ~ 80 km from the main component, respectively, and their radial extent is indicated by the shaded region in Fig. 1a, b.

It is clear from Fig. 1b that an object the size of Prometheus would be intersecting both the core and first inner strand of the F ring for the configuration thought to have existed in February 1994. Although the orbit of Pandora is not plotted, its gravitational effect was included in the calculation. We find that the eccentricity of the F ring varies from 0.00257 to 0.00288 with a period $T = 19.08$ yr. The resulting value of Δ has the same period with minima of $\Delta_{\min} = 59$ km occurring in February 1994 and March 2013 (Fig. 2).

There are a number of uncertainties in the orbital elements (Table 1), principally in the value of $\bar{\omega}$ for each object. Although errors in $\bar{\omega}$ affect neither T nor $\Delta_{\min}$, they do give rise to an uncertainty of $\sim \pm 1.7$ yr in the time at which $\Delta_{\min}$ occurs¹⁴. Altering the eccentricities does not affect the period, but decreases

ing them by $\delta e = 0.006$ gives $\Delta_{\min} = 236$ km. A corresponding increase by δe would produce actual intersection of the orbits in January 1991. The uncertainty in the semimajor axis of the F ring and secular perturbations from other satellites have a negligible effect. If the densities of Prometheus and Pandora are as low as those of Janus and Epimetheus (~ 0.65 g cm⁻³; ref. 7) this would give $\Delta_{\min} \approx 80$ km with a lower T . If the orbits have inclinations as large as 0.1° (see Table 1) then the value of $\Delta_{\min}$ will also depend on the relative location of the nodes of the orbits and $\Delta_{\min}$ will be larger than in the planar case with larger values of T . However, for a wide range of starting conditions there should always be an eventual intersection of Prometheus with part of the F ring. We estimate that the inner strand and the core are crossed by part of Prometheus within ± 2.3 yr and ± 0.76 yr respectively of the time of $\Delta_{\min}$.

It is conceivable that the F ring could contain a total mass comparable to that of Prometheus^{15,16}. Using the secular theory we have investigated the relationship between the assumed mass of the F ring and the value of $\Delta_{\min}$. Our calculations show that only if the mass is >2.5 times that of Prometheus can all collisions be avoided for a coplanar system (Fig. 3). Such a mass is unlikely as there would be detectable perturbations on Pandora's orbit.

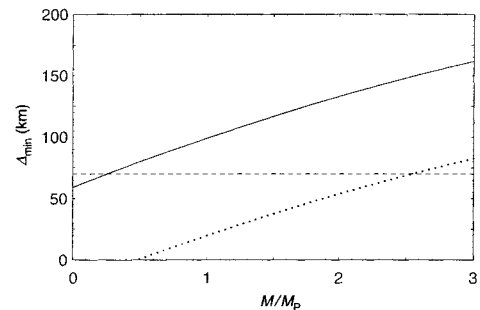


FIG. 3 The variation of the minimum distance of closest approach, $\Delta_{\min}$, between Prometheus and the main component of the F ring as a function of the mass of the F ring. This mass is expressed in units of the mass of Prometheus (M_p) using the value given in Table 1. The dashed line denotes a distance equal to the longest radius of Prometheus. The dotted line denotes the equivalent value of $\Delta_{\min}$ for the inner strand.

Furthermore, encounters with the core seem certain to occur unless the mass of the F ring is $>25\%$ that of Prometheus.

The effect on the orbit of Prometheus of an object striking that moon can be modelled by assuming that all collisions take place in the $\Delta_{\min}$ configuration with Prometheus at apoapse and the object at periapse. In these circumstances there is a change in semimajor axis given by

$$\delta a \approx (m'/m)[2a(e+e') - (a' - a)] \quad (2)$$

where m , a and e denote the mass, semimajor axis and eccentricity of Prometheus and the primed quantities refer to the object that strikes Prometheus. Note that $\delta a > 0$ for parameters appropriate to the Prometheus-F ring system. From Kepler's third law the corresponding change in the mean motion n (the average angular velocity of Prometheus) is $\delta n = -(3/2)(n/a)\delta a$. If t is the time interval since the collision, then the difference between the expected and actual longitudes of Prometheus is given by $\delta \lambda = \delta n \times t$. Currently $\delta \lambda \approx -20^\circ$, implying that if the collision took place in February 1994 then the impactor must have had a mass $\sim 1\%$ of the mass of Prometheus. Although there is some suggestion that objects of

TABLE 1 Properties of Prometheus, the F ring and Pandora

Parameter	Prometheus	F ring	Pandora
a (km)	139,377	$140,175 \pm 32$	141,712
i ($^\circ$)	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1
e	$(2.4 \pm 0.6) \times 10^{-3}$	$(2.6 \pm 0.6) \times 10^{-3}$	$(4.2 \pm 0.6) \times 10^{-3}$
$\bar{\omega}$ ($^\circ$)	173 ± 20	230 ± 15	22 ± 30
n ($^\circ$ d ⁻¹)	587.2890 ± 0.0005	582.27 ± 0.2	572.7891 ± 0.0005
D (km)	$140 \times 100 \times 75$	—	$110 \times 85 \times 65$
m/M_s	1.15×10^{-9}	—	7.66×10^{-10}

a is the semimajor axis, i is the inclination, e is the eccentricity, $\bar{\omega}$ is the longitude of pericentre, n is the mean motion, D is the diameter, and m/M_s is the mass given in units of Saturn's mass using the stated diameters and an assumed satellite density of 1.2 g cm⁻³. The epoch is 04:00 UTC on 23 August 1981. The orbital elements are taken from Synnott et al.¹⁰ with the exception of the semimajor axes which are calculated from the mean motions and include the effects of Saturn's first two zonal gravity coefficients, J_2 and J_4 . The diameters are taken from Thomas et al.¹⁷.

~10 km radius exist in the F ring, we consider the impact of such a large object in February 1994 to be unlikely. One reason for this is that there should have been a detectable increase of 3° in the observed lag angle between the May and August 1995 detections of Prometheus. An alternative explanation is that a single impact with a smaller object occurred earlier in the region of the inner strands, or perhaps there have been several smaller impacts during the current series of encounters. We also note that an error of only $4 \times 10^{-3}^\circ \text{d}^{-1}$ in the mean motion of Prometheus (~8 times the stated error in Table 1) would also produce the observed lag but with only 0.3° change in $\delta\lambda$ between ring plane crossing detections. Astrometric observations of Prometheus obtained during 1995 and 1996, together with a reanalysis of the Voyager data, could help to distinguish between these hypotheses.

The August 1995 observations revealed the existence of an object, S/1995 S 7, in the same orbit as Prometheus, but trailing it by 15° (ref. 6). Such an object is too close to Prometheus to be moving in a Trojan-like 'tadpole' orbit around the trailing lagrangian equilibrium point, L_5 , but if their orbits are separated by <100 km then the object must be following a 'horseshoe' orbit encompassing Prometheus's L_3 , L_4 and L_5 points. (The terms 'tadpole' and 'horseshoe' refer to the path of the smaller body as viewed in a reference frame rotating with the mean motion of the larger body.) This is similar to the configuration known to exist for the saturnian moons Janus and Epimetheus⁷ where the orbit of each changes by $\delta a_1 = \pm 10$ km and $\delta a_E = \mp 40$ km respectively every four years with $\delta a_1/\delta a_E = m_E/m_J$, the ratio of the masses. If the stated equivalent radius of 18 km for S/1995 S 7 (ref. 6) is that of a solid body with the same density as Prometheus (as opposed to a cloud from a collision), then it is possible that periodic encounters could lead to similar changes in the orbit of Prometheus and S/1995 S 7, the exact magnitude of the changes depending on the current separation in semimajor axis. In any case, if the object is as massive as its brightness suggests, its effect on Prometheus could be significant. One advantage of this model is that the lag in Prometheus's longitude will increase only during those times when Prometheus has a semimajor axis larger than its Voyager value; therefore observations of a constant lag in 1995 would imply that its current semimajor axis is similar to the value it had in 1981. However, further information about the orbit and physical properties of S/1995 S 7 must be obtained before Prometheus's companion can be considered as a possible cause of the lag in its position.

Our results suggest that unless the F ring has an unrealistically large mass or relative inclination, it is likely that Prometheus collides with it every ~19 yr. This represents the only known example of direct impacts of macroscopic ring material on to the surface of a satellite. Preliminary studies^{8,14} have shown that the perturbations on the F ring produce severe disruption and that azimuthal gaps may form. The availability of better information on the orbital parameters of all the objects involved in this unusual dynamical system may have to await the arrival of the Cassini orbiter at Saturn in 2004. But with an orbital tour that finishes in 2008 the present evidence suggests that unless there is an extended mission, Cassini will be too late to witness the current series of encounters and too early to witness the next. □

Received 25 October 1995; accepted 18 January 1996.

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ACKNOWLEDGEMENTS. We thank M. Gordon and K. Beurle for their help with Voyager image analysis, and D. Hamilton and M. Showalter for suggesting improvements to the original manuscript. C.D.M. is grateful to the PPARC for an Advanced Fellowship, and S.M.G.W. thanks CAPES and CNPq for financial support.

Giant magnetoresistance of manganese oxides with a layered perovskite structure

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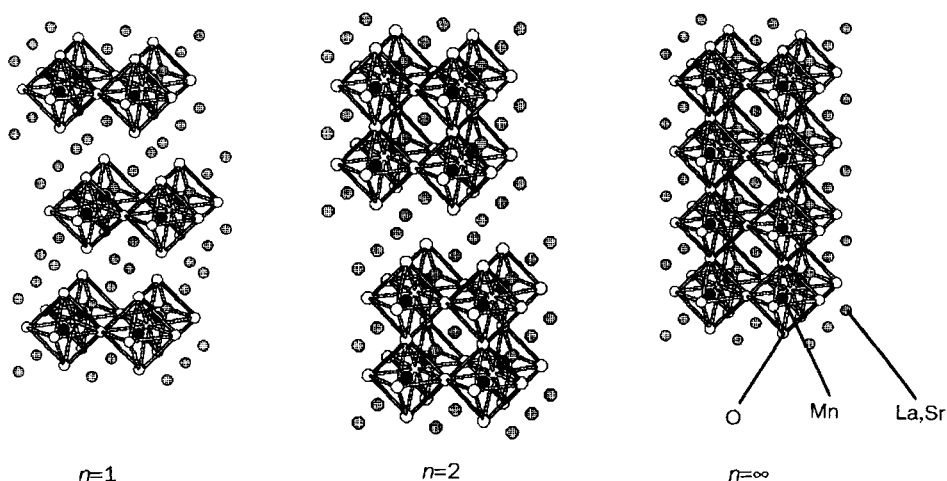
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MANGANESE oxides with the cubic perovskite structure (typified by LaMnO_3) have stimulated considerable interest because of their magnetoresistive properties^{1–9}; they exhibit extremely large changes in electrical resistance in response to applied magnetic fields, a property that is of technological relevance for the development of magnetic memory and switching devices. But for such applications to be viable, great improvements will be needed in both the sensitivity and temperature dependence of the magnetoresistive response. One approach under consideration for optimizing these properties is chemical substitution¹⁰. Here we demonstrate an alternative strategy, in which we synthesize layered variants of the cubic perovskite parent compounds that have a controlled number of MnO_2 sheets per unit cell. This strategy is structurally analogous to that employed for the systematic exploration of the high-transition-temperature copper oxide superconductors¹¹. We find that the magnetoresistive properties of these materials depend sensitively on the dimensionality of the manganese oxide lattice. Although the properties of our materials are still far from optimal, further exploration of this series of layered perovskites may prove fruitful.

The ferromagnetic ground state of metallic $\text{La}_{1-x}\text{Sr}_x\text{MnO}_3$ is caused by the so-called double-exchange interaction between the Mn^{3+} and the Mn^{4+} ions^{12–14}. The parent antiferromagnetic insulator LaMnO_3 contains Mn^{3+} ions with $t_{2g}e_g^1$ (spin quantum number $S = 2$) configuration. The t_{2g} electrons are localized and so can be viewed as local spins with $S = 3/2$. In contrast, the e_g electrons are strongly hybridized with the oxygen $2p$ states and change in character from localized to itinerant with oxidation of the MnO_3 lattice¹². An important feature is that strong intra-atomic ferromagnetic (Hund) coupling acts between the t_{2g} spins and e_g electrons. Chemically doped e_g carriers mediate a ferromagnetic interaction between the local Mn spins and cause the ferromagnetism. The giant magnetoresistive (MR) effect observed in $\text{La}_{1-x}\text{Sr}_x\text{MnO}_3$ (refs 6, 7) can be interpreted in terms of such a double-exchange mechanism. Near the ferromagnetic ordering temperature (T_c), an external magnetic field aligns the t_{2g} spins and reduces the carrier scattering by the local spins.

We can modify the perovskite-type structure, $(\text{La}, \text{Sr})\text{MnO}_3$, into layered structures by inserting a rock-salt-type block layer $((\text{La}, \text{Sr})_2\text{O}_2)$ every n MnO_2 -sheets; that is $(\text{La}, \text{Sr})_{n+1}\text{Mn}_n\text{O}_{3n+1}$. Schematic structures of the layered family are shown in Fig. 1 for the single- ($n = 1$; K_2NiF_4 structure), double- ($n = 2$) and ∞ -sheet (cubic perovskite) compounds. Nominal hole concentration (x) can be controlled by substitution of the La sites with Sr in the form of $\text{La}_{1-x}\text{Sr}_x\text{MnO}_4$ ($n = 1$), $\text{La}_{2-2x}\text{Sr}_{1+2x}\text{Mn}_2\text{O}_7$ ($n = 2$) and $\text{La}_{1-x}\text{Sr}_x\text{MnO}_3$ ($n = \infty$). The single-sheet ($n = 1$) compound is not metallic or ferromagnetic¹⁵. With increasing n , however, the

FIG. 1 Schematic structures of the Mn-based layered perovskite $(\text{La}, \text{Sr})_{n+1}\text{Mn}_n\text{O}_{3n+1}$ with $n = 1, 2$ and ∞ (cubic perovskite). The MnO_6 octahedra are shown in polyhedral representation. With the nominal hole concentration of $x = 0.4$, the $n = 1$ and $n = 2$ crystals belong to tetragonal space group ($I4/mmm$, $Z = 2$) and the $n = \infty$ crystal to a rhombohedral space group ($R\bar{3}c$, $Z = 2$). The room-temperature lattice parameters are $a = b = 3.86(1) \text{ \AA}$ and $c = 12.48(8) \text{ \AA}$ for $n = 1$, $a = b = 3.87(9) \text{ \AA}$ and $c = 20.14(4) \text{ \AA}$ for $n = 2$, and $a = 5.45(8) \text{ \AA}$ and $\alpha = 60.05(3)^\circ$ for $n = \infty$.



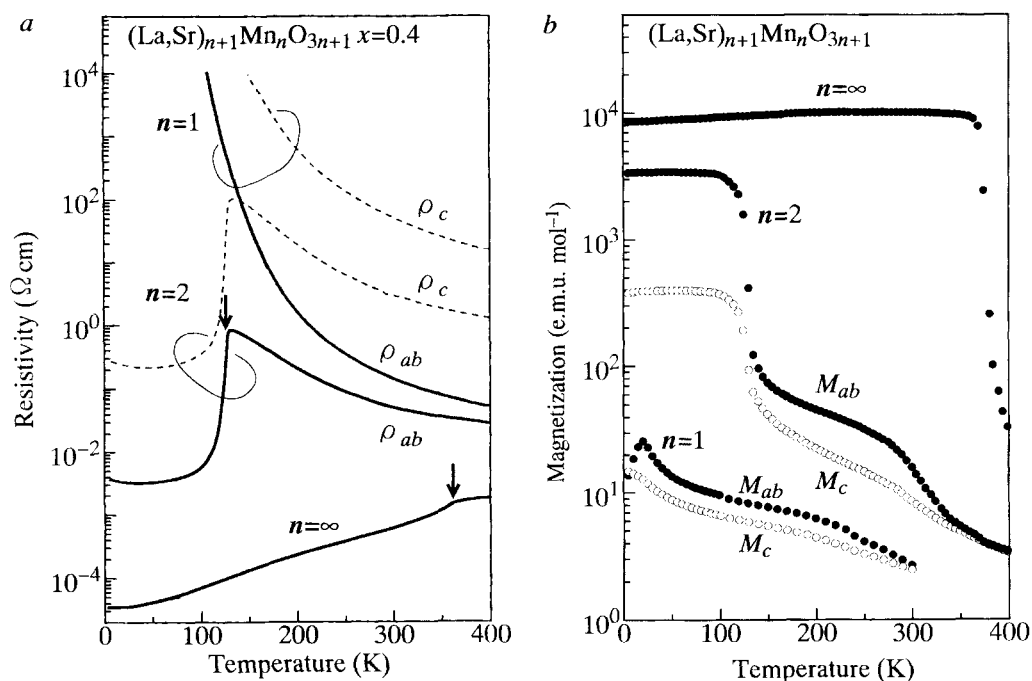
electronic and magnetic properties should approach those of the $n = \infty$ compound. Indeed, the double-sheet ($n = 2$) compound, $(\text{La}, \text{Sr})_3\text{Mn}_2\text{O}_7$, is found to be a ferromagnetic metal below $T_c = 126 \text{ K}$, and exhibits large negative magnetoresistance without hysteresis above T_c .

A single crystal of $(\text{La}_{0.4}\text{Sr}_{0.6})_3\text{Mn}_2\text{O}_7$ ($x = 0.4$) was grown in a flow of air by a floating-zone method. A typical size of the grown crystal is 3.5 mm diameter, 10 mm long. The crystal can be cleaved parallel to the growth direction, yielding the (001) surface. (Single crystals with $n = 1$ and ∞ were also melt-grown by a similar method under the growth conditions described in refs 6, 7, 16). Electron-probe microanalysis indicated a homogeneous composition within the crystal. The powder X-ray diffraction was consistent with a body-centred tetragonal unit cell ($I4/mmm$; $Z = 2$). The room-temperature lattice parameters are $a = b = 0.387 \text{ nm}$ and $c = 2.014 \text{ nm}$, in good agreement with published results¹⁵ on the polycrystalline compound.

Figure 2a shows the temperature dependence of electrical

resistivity for the layered ($n = 1$ and 2) and pseudo-cubic ($n = \infty$) compounds; the current parallel (ρ_{ab} ; solid curves) and perpendicular (ρ_c ; dotted curves) to the layer is shown. The nominal hole concentration is controlled to be $x = 0.4$, where the $n = 2$ and ∞ compounds show the highest T_c values (refs 6, 7, 12). A sharp drop of the resistivity by more than two orders of magnitude is observed around $T_c = 126 \text{ K}$ in the double-sheet ($n = 2$) compound. The variation of the resistivity with temperature for $T \geq 150 \text{ K}$ obeys the thermal activation law; the activation energy is slightly larger in ρ_c ($\sim 40 \text{ meV}$) than in ρ_{ab} ($\sim 30 \text{ meV}$). A steep rise of the magnetization (Fig. 2b) observed around T_c indicates a phase change from a paramagnetic to ferromagnetic state. Even in the ferromagnetic metal phase, motion of the charge carriers appears to be highly anisotropic: ρ_c is $\sim 10^2$ times as large as ρ_{ab} . Anisotropy of the low-field (10-mT) magnetization ($M_{ab}/M_c \approx 10$) indicates that the easy axis lies in the Mn-O layer. No remanent magnetization is observed in the low-temperature ferromagnetic phase, similar to the $n = \infty$ compound^{6,7}. In

FIG. 2 Temperature dependence of resistivity (a) and magnetization (b) for members of layered perovskite family, $(\text{La}, \text{Sr})_{n+1}\text{Mn}_n\text{O}_{3n+1}$ ($n = 1, 2$ and ∞). The nominal hole concentration is controlled to be $x = 0.4$. Anisotropy is shown for the $n = 1$ and 2 crystals. Solid and dashed curves are the resistivity with the current parallel (ρ_{ab}) and perpendicular (ρ_c) to the layer, respectively. A fairly large ($\sim 2 \times 3(ab) \times 2(c) \text{ mm}$) single-crystal specimen enabled us to measure ρ_c as well as ρ_{ab} by the conventional four-probe method. The critical temperature (T_c) for the ferromagnetic phase transition was determined to be 126 K for $n = 2$ and 361 K for $n = \infty$ by measurements of a.c. susceptibility, and is marked with an arrow. Filled and open circles represent the magnetization with the magnetic field parallel (M_{ab}) and perpendicular (M_c) to the layer. Magnetization was measured in the warming run with a field of 10 mT after cooling down to 5 K in zero field using a superconducting quantum interference device (SQUID) magnetometer.



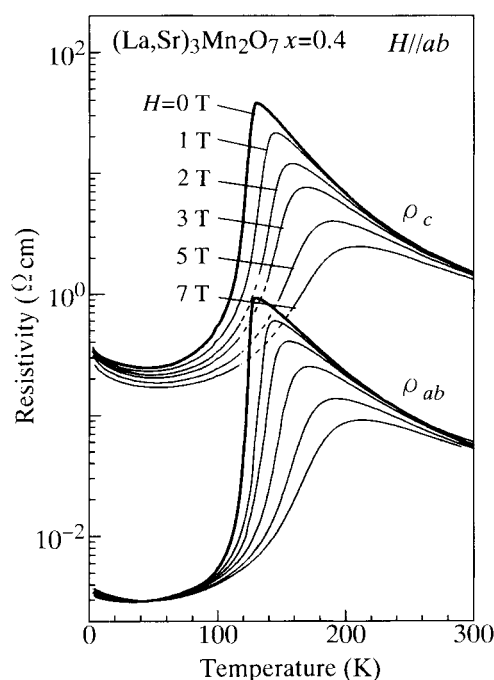


FIG. 3 Temperature dependence of resistivity for single crystal of the $n = 2$ compound, $(\text{La}_{0.4}\text{Sr}_{0.6})_3\text{Mn}_2\text{O}_7$ (with the nominal hole concentration of $x = 0.4$), with an external magnetic field applied parallel to the layer ($H \parallel ab$). Thick lines represent the resistivity without field. The lower and upper sets of curves correspond to the resistivity with the current parallel (ρ_{ab}) and perpendicular (ρ_c) to the layer, respectively. No thermal hysteresis was observed.

contrast to the $n = 2$ compound, in the $n = \infty$ compound the metallic region extends over the whole temperature range. The ferromagnetic phase transition shows up as a small drop-off of the resistivity at ~ 361 K (indicated by an arrow) in Fig. 2a (refs 6, 7). On the other hand, the $n = 1$ compound, with an isolated MnO_2 sheet, is insulating without ferromagnetic ordering. A cusp structure observed at ~ 20 K in the M_{ab} - T curve corresponds to a spin-glass transition¹⁶.

Very large negative MR was observed for the $n = 2$ compound just above $T_c = 126$ K for the configurations with the current both parallel (ρ_{ab}) and perpendicular (ρ_c) to the layer (Fig. 3). A large single-crystal specimen enabled us to measure easily and precisely the MR with the current perpendicular to the layer. The observed negative MR can be interpreted in a manner qualitatively similar to the case of the $n = \infty$ compound^{6,7}: an applied magnetic field aligns the t_{2g} spins and reduces scattering of the carriers by the local spins. However, the reduced dimensionality of the Mn-O network appears to dramatically enhance the MR effect. As seen in the upper panel of Fig. 4, the resistivity rapidly decreases with a relatively low magnetic field (≤ 1 T). At 129 K, the MR ratio (as defined by $\rho(0)/\rho(H)$) reached $\sim 200\%$ at 0.3 T, $\sim 3,000\%$ at 1 T and increased up to $\sim 20,000\%$ at 7 T. This is in sharp contrast to the $n = \infty$ system, $(\text{La}, \text{Sr})\text{MnO}_3$, in which the change of the resistivity is rather gradual with field change; $\rho(0)/\rho(H) \approx 110\%$ at 1 T and $\sim 2,000\%$ at $H = 15$ T around T_c , even when the composition x is optimized (~ 0.15) so as to product the highest MR value^{6,7}.

The enhanced MR property in the present compound is partly due to the anisotropic exchange interaction. The intra-layer exchange interaction is obviously due to the double-exchange interaction, and is perhaps much stronger than the inter-layer value that contributes to the actual three-dimensional magnetic ordering. Reflecting such a high anisotropy of the exchange interaction, two-dimensional spin correlation is maintained in the paramagnetic phase ($T \geq T_c$). An external magnetic field

then increases the magnetization (Fig. 4, lower panel) and perhaps reduces the spin scattering of the charge carriers. Another possible origin of the much-enhanced MR effect is reduction in the effective transfer interaction of the e_g carriers, which is caused by the reduced dimensionality as well as by the lifting of the e_g state degeneracy in the layered structure. As a result, the doped carriers are strongly localized in the spin-disordered paramagnetic phase, as observed in the thermal-activation type behaviour of resistivity above T_c (Fig. 2a). Just above T_c , the $n = 2$ compound may be further subject to other competing instabilities with the double-exchange interaction, such as the antiferromagnetic superexchange, electron-lattice (for example, the Jahn-Teller effect¹⁷) and charge-ordering interactions^{8,9}. In this sense, the MR phenomena observed here may be interpreted as a field-induced insulator-to-metal transition. In accord with such a feature, the magnetization curve shows a nonlinear enhancement (compare with dashed lines representing the initial slopes of the magnetization curve in Fig. 4) at around the field where the metallic conduction begins to appear.

The Mn-based layered perovskite with the $n = 2$ structure shows an extremely large MR effect, which is by far superior to that of the cubic ($n = \infty$) system. The MR phenomenon shows no hysteresis with an external magnetic field, in spite of a huge resistivity change (more than two orders of magnitude). As noted when comparing the $n = 2$ and $n = \infty$ compounds, the improved MR is attained at the cost of decreased T_c owing to the reduction of the kinetic energy of the carriers. In other words, the trade-off relation between attaining higher T_c and lowering operating field might pose an obstacle to the development of the technologically useful materials based on the layered perovskites. Nevertheless,

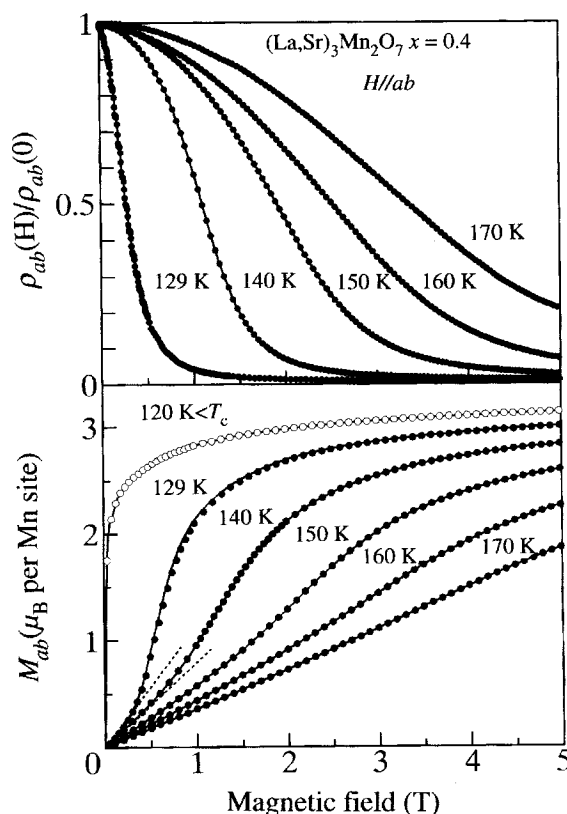


FIG. 4 Top panel, change of the in-plane component of resistivity (ρ_{ab}) with increasing magnetic field for a single crystal of $(\text{La}_{0.4}\text{Sr}_{0.6})_3\text{Mn}_2\text{O}_7$. Magnetic field is parallel to the layer ($H \parallel ab$). No hysteresis was observed with increasing and decreasing the magnetic field. Bottom panel, field-induced magnetization (in-plane component, M_{ab}). Open and filled circles are the data in the ferromagnetic ($T \leq T_c$) and paramagnetic ($T \geq T_c$) phases. Dotted lines represent the initial slopes of the M - H curves.

the reduced electronic-dimensionality in the layered perovskite may cause larger magnetic fluctuations up to temperatures significantly above T_c , which should favour the MR characteristics as demonstrated here for the $n = 2$ compound. Further control of the MnO_2 -sheet number ($n \geq 3$) in the layered perovskites, for example by use of the molecular beam epitaxy method, should help toward achieving useful MR characteristics at the desired operating conditions of room temperature and low field. $\square$

Received 6 March 1995; accepted 26 January 1996.

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ACKNOWLEDGEMENTS. We thank Y. Yokoyama for her help with the electron-probe microanalysis. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

Intermolecular interactions in the molecular ferromagnetic $\text{NH}_4\text{Ni}(\text{mnt})_2 \cdot \text{H}_2\text{O}$

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MOLECULAR solids that exhibit ferromagnetism are rare, and thus there is considerable interest in understanding the magnetic coupling mechanisms that operate in the few known examples¹. One such material is the charge-transfer salt $\text{NH}_4\text{Ni}(\text{mnt})_2 \cdot \text{H}_2\text{O}$, which consists of stacked planar metal ligands separated by ammonium cations. This salt is an insulator with localized spins that exhibit long-range ferromagnetic order at low temperatures (below 4.5 K)². Here we show that the Curie temperature demarcating the transition to the ferromagnetic state increases markedly with pressure until ferromagnetic order abruptly disappears at 6.8 kbar, indicating that the magnetic coupling is very sensitive to intermolecular separation. Using quantum-chemical calculations³, we show that this pressure dependence arises from a competition between ferromagnetic coupling (resulting from nickel–sulphur intermolecular spin interactions), and antiferromagnetic coupling (from nickel–nickel interactions). We suggest that a similar interplay of spin-polarization effects might play a key role in determining the nature of the ground states (metallic, superconducting and so forth) observed in other molecular materials of this structural type^{4,5}.

The structure of the nickel bis(maleonitrile) complex, $\text{Ni}(\text{mnt})_2^-$ is shown in Fig. 1. Charge transfer salts of the type $\text{M}'\text{M}(\text{mnt})_2$, which have M' and $\text{M}(\text{mnt})_2$ in a 1:1 stoichiometry and form segregated stacks of donor and acceptor, are usually found to be dimerized, insulating and antiferromagnetic^{6,7}. The ammonium salt of the $\text{Ni}(\text{mnt})_2^-$ complex is therefore unusual in that it is undimerized at room temperature⁸. Although only a single crystal structure is found, both antiferromagnetic^{9,10} and ferromagnetic² properties have been reported. The different properties are correlated with the details of the sample preparation method¹¹. $\text{NH}_4\text{Ni}(\text{mnt})_2 \cdot \text{H}_2\text{O}$ was prepared by electrolysis between two platinum electrodes in a solution of urea and $[\text{NH}_4]_2[\text{Ni}(\text{mnt})_2]$ in deionized, degassed water under a nitrogen atmosphere at 1.0 V. Crystals grew as black platelets over a period of 8 days, and X-ray measurements indicate that the unit cell of the material investigated here is the same as that prepared previously².

Previously measurements of randomly oriented $\text{NH}_4\text{Ni}(\text{mnt})_2 \cdot \text{H}_2\text{O}$ crystals show a magnetic susceptibility which indicates antiferromagnetic coupling above 100 K, but ferromagnetic coupling below this temperature, with a Weiss constant of 7 K and a Curie constant consistent with $0.4s = 1/2$, $g = 2$ spins per formula unit². At lower temperatures, the susceptibility increases rapidly, indicating the presence of ferromagnetic order below 5 K. We have measured small single crystals in a Quantum Design SQUID magnetometer down to 2 K. As shown in Fig. 2, magnetization curves were taken with the long axis of the crystal (established to be the stacking axis²) both parallel and perpendicular to the applied field. It is evident from the figure that the magnetization response is strongly anisotropic at this temperature (2 K), with the hard axis parallel to the stacking axis. This anisotropic magnetization response is direct evidence for long-range ferromagnetic order. For fields applied perpendicular to the stacking axis, hysteresis is seen, with a coercive field of around 100 oersted, and a magnetic moment of $1,800 \text{ e.m.u. G mol}^{-1}$ is measured at 2,000 oersted. If we take a value of $g = 2$ for $\text{NH}_4\text{Ni}(\text{mnt})_2 \cdot \text{H}_2\text{O}$, then this saturation magnetization corresponds to around one third of a spin $1/2$ electron per $\text{Ni}(\text{mnt})_2$ complex. We note that this matches the number of spins present in the paramagnetic phase below 100 K measured on polycrystalline samples².

The a.c. magnetic susceptibility was measured under hydrostatic pressure using a small coil around the sample placed inside the pressure cell¹². Changes in the resonant frequency of this coil in combination with a capacitor placed next to the pressure cell were measured. The ambient pressure response of the system to a sample of $\text{NH}_4\text{Ni}(\text{mnt})_2 \cdot \text{H}_2\text{O}$ is shown in Fig. 3. The peak at 4.5 K corresponds to the peak in susceptibility at the Curie temperature. The Curie temperature rises very rapidly with pressure, at 0.4 K kbar^{-1} , as is evident in Fig. 3 from the response at 6.8 kbar. Measurements on samples from several batches, made with capillary-pressure delivery and with clamp pressure cells¹², are

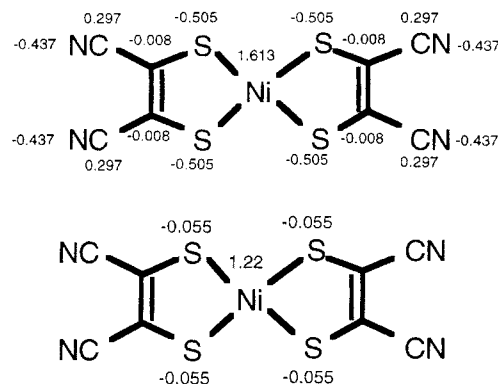


FIG. 1 Structure of the $\text{Ni}(\text{mnt})_2^-$ anion. Shown also are the results of the UHF/INDO calculations for charge (top) and spin density (bottom) distributions.

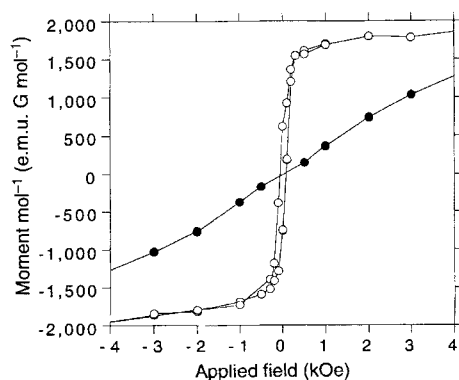


FIG. 2 Magnetization curves of a 11- μ g single crystal of $\text{NH}_4\text{Ni}(\text{mnt})_2 \cdot \text{H}_2\text{O}$ taken at 2 K. Solid data points were measured with the applied field parallel to the b -axis (stacking axis); open circles, the applied field was perpendicular (kOe, kilo-oersted).

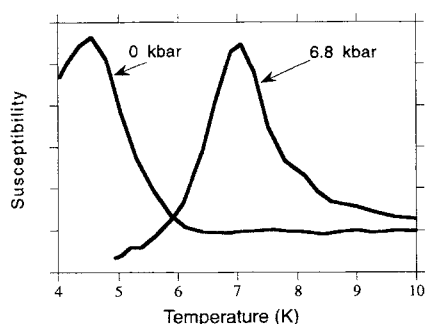


FIG. 3 The a.c. susceptibility response of $\text{NH}_4\text{Ni}(\text{mnt})_2 \cdot \text{H}_2\text{O}$ at atmospheric pressure and with 6.8 kbar of pressure applied.

summarized in Fig. 4. Measurements taken at and above a pressure of 7.4 kbar show no indication for a ferromagnetic transition, and the ferromagnetic response was only re-established after the samples had been returned to ambient pressure. We consider that there is a structural phase transition at this pressure to a metastable phase which reverts back to the low-pressure phase only at low pressures.

These results provide confirmation of ferromagnetic ordering in this material, and evidence for an exceptionally large pressure dependence of the Curie temperature. We note that this is twice the value found for the molecular ferromagnet decamethylferrocenium tetracyanoethanide¹³. We have investigated the temperature and pressure dependence of the magnetic properties by using the UHF/INDO³ and RHF/INDO/SD-CI quantum chemical techniques. The change from antiferromagnetic to ferromagnetic behaviour is described in terms of the singlet-triplet energy separation for a bilayer. At both the UHF and RHF/CI levels, we find that there is a singlet-triplet (that is, antiferromagnetic to ferromagnetic) crossing as the distance between the two molecular units is reduced (Fig. 5); we associate this with a switch from dominant metal-metal interactions to dominant metal-sulphur interactions (note that, in all calculations, we keep the starting room temperature slipped configuration). The loss of ferromagnetic order above 6.8 kbar is associated with a structural phase transition, and we do not at present know the structure of the high-pressure phase. We note, however, that relatively small adjustments of the intermolecular arrangement, such as a switch to an eclipsed rather than staggered arrangement, can easily reverse the sign of the magnetic coupling.

The origin of the stabilization of the ferromagnetic interaction (triplet dimer state) for certain intermolecular contact distances can be illustrated qualitatively by means of McConnell's theory¹⁴, based on spin polarization effects. Following this theory, ferromagnetic coupling requires two conditions to be met: first, non-

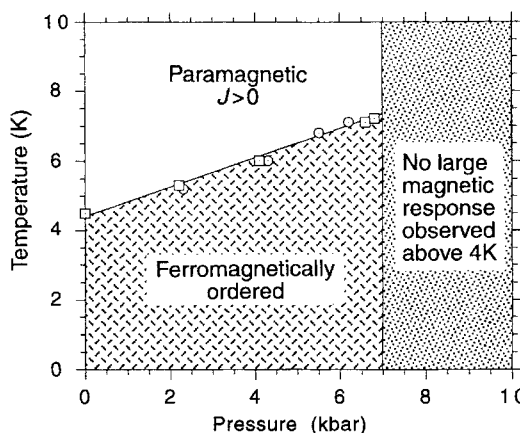


FIG. 4 Magnetic phase diagram of $\text{NH}_4\text{Ni}(\text{mnt})_2 \cdot \text{H}_2\text{O}$ with temperature and pressure. Squares denote ordering temperatures measured in a clamp type pressure cell and circles denote those measured in a capillary cell system¹².

compensating regions of positive and negative spin densities, here distributed over the atomic sites of the $\text{Ni}(\text{mnt})_2$ complex, and second, the assembling of the units in an 'up-down' fashion (namely in such a way that the positive spin density of a unit interacts with the negative spin density of the adjacent unit). By way of illustration, we present in Fig. 1 the charge and spin distributions for a single $\text{Ni}(\text{mnt})_2^-$ anion, as calculated at the UHF/INDO level. (Calculation of the spin density at the ROHF/CI level is much more challenging: the UHF/INDO technique we use has been shown successfully to reproduce the electronic structure of a wide range of transition metal complexes^{15,16} and to provide a qualitatively reliable description of the spin distribution¹⁷.) A large positive spin density, due to excess of spin-up electrons with respect to spin-down electrons, is calculated on the nickel atom, whereas the sulphur atoms share a smaller negative spin density. Furthermore, the $\text{Ni}(\text{mnt})_2^-$ layers are superimposed in a slipped metal-over-sulphur configuration within the stacks⁸, and we note that this does therefore give the 'up-down' arrangement of the spin densities along the stacking axis which is the second condition in the McConnell model for ferromagnetic coupling.

A wide range of charge-transfer salts containing planar metal complexes of this type have been investigated, which can show metallic^{18,19} and superconducting²⁰⁻²³ behaviour. The strong spin polarization that is seen in the UHF/INDO calculations here is not of course evident in the one-electron calculations of band

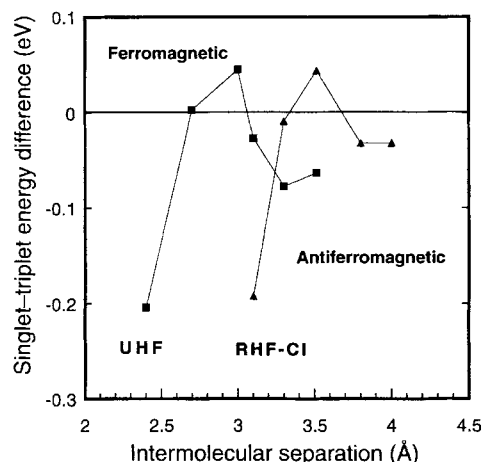


FIG. 5 Variation of the singlet-triplet energy difference for a dimer of $\text{Ni}(\text{mnt})_2$ as a function of the intermolecular separation.

schemes that have been widely used^{18,24}. In the particular case of the compound studied here, which is a Mott–Hubbard insulator, the intramolecular spin polarization is manifest in the appearance of ferromagnetic order. Segregated-stack compounds with stronger intermolecular contacts along the stack are metals at room temperature (rather than Mott–Hubbard insulators, as here) but display a wide range of ground states at low temperatures (superconducting, spin density wave, Peierls insulators, spin-Peierls insulators), on account of the instability of the quasi-one-dimensional electron gas to these symmetry-breaking ground states. We consider that the mechanism we have identified here for ferromagnetic coupling can, if the intermolecular arrangements in the molecular stack are correct, reduce or reverse the antiferromagnetic interactions that are generally found to be strong (antiferromagnetic coupling strengths of around 100 K; ref. 25). This may be important to allow the metallic or superconducting states to appear at low temperatures. For example, the TMTSF Bechgaard salts (where TMTSF is tetramethyltetraselenafulvalene) are weakly antiferromagnetic at ambient pressure but superconducting at modest pressures⁴. In this context, we note that there is a complex interplay between weak ferromagnetism and superconductivity in κ -(ET)₂Cu[N(CN)₂]Cl, where ET is bis(ethylenedithio)-tetrathiafulvalene^{26,27}. □

Received 17 October 1995; accepted 1 February 1996.

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ACKNOWLEDGEMENTS. We thank the Engineering and Physical Sciences Research Council, and the European Commission, through a Human Capital Mobility Network Grant, for support. The work in Mons is partly supported by the Belgian Prime Minister's Office of Science Policy "Pôle d'Attraction Interuniversitaire en Chimie Supramoléculaire", the Fonds National Belge de la Recherche Scientifique, and an IBM Academic Joint Study grant.

Magma fragmentation by rapid decompression

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THE processes leading to magma fragmentation and the generation of pyroclastic debris during explosive volcanic eruptions are of fundamental importance in volcanology. Observations of explosive eruptions^{1–3}, as well as theoretical analyses of the underlying processes⁴, have raised the question of whether the rapid decompression of highly viscous, vesicular magma that results from the collapse of a lava dome or volcanic edifice can, in itself, produce explosive magma fragmentation and pyroclast formation. Here we report the results of a laboratory investigation of pyroclast formation following rapid decompression. Samples of magma from the 1980 eruption of Mount St Helens, when rapidly depressurized from initial pressures of up to 12 MPa (and at temperatures in the range 750–825 °C), fragmented to form pyroclastic products that are in many respects similar to those formed in real eruptions. Moreover, we observe explosive fragmentation at temperatures well below those normally associated with magmatic processes, suggesting that even relatively cool magma bodies can be very hazardous when subjected to rapid unloading events.

During eruptions, interiors of lava domes and cryptodomes can undergo rapid decompression owing to either the partial collapse of lava domes or landslides involving the volcanic edifice. Quantification of the material parameters pertaining to such processes is a prerequisite for experimental analysis of dome collapse. Estimates of relevant material parameters include the viscosity of the dacite lava dome (1991 eruption of Unzen), 10⁸ Pa s (for 2 wt% water) to 10¹¹ Pa s (water-free) at 800 °C (ref. 3); surface crust strength (Mount St Helens dacite dome, 0.1–10 MPa (ref. 4); tensile strength of andesite at subsolidus temperatures, 10–20 MPa (ref. 5); lava-dome water content, 0.1–0.5 wt% (ref. 4);

and initial gas pressures ≤ 20 MPa (1980 Mount St Helens cryptodome)⁶. The present experiments were designed with these estimates of material and intensive parameters in mind.

Our experimental shock-tube-type apparatus operates on some of the same principles as facilities previously employed in laboratory simulations of magma fragmentation using analogue materials^{7–9} but differs in several fundamental aspects. It consists of a high-pressure/high-temperature section and a low-pressure/low-temperature section separated by a steel diaphragm. Pressurized argon gas fills the open pores of the sample and the space between the sample surface and the diaphragm. The essential details are shown in Fig. 1. The ratio of volumes of the high- and low-pressure sections (~10⁻⁴) is sufficiently small to ensure near-isobaric conditions following outburst into the low-pressure tank.

Disruption of the steel diaphragm (which has been scored with a pattern to ensure regular, reproducible rupture behaviour) occurs at a certain pressure differential between the high- and low-pressure sections, causing the propagation of a shock wave into the low-pressure section. The head of a rarefaction wave propagates into the high-pressure section containing the sample¹⁰. This wave strikes the upper surface of the sample and is reflected, resulting in a pressure drop on the sample surface. The pressure history of the surface of the sample is a function of the initial pressures in the high-pressure and low-pressure sections, gas parameters and the geometry of the sections. Computer simulation of the gas dynamical flow inside the shock-tube-type apparatus, with an open-ended low-pressure section, was performed using the software KASIMIR of the Stosswellenlabor, RWTH Aachen, which simulates one-dimensional flow following instantaneous diaphragm disruption. (The effects of a finite disruption time have yet to be quantified.) For the case of Ar at 12 MPa and 825 °C in the high-pressure section (length 210 mm) and air at 0.1 MPa and 20 °C in the low-pressure section (length 120 mm, open ended), membrane rupture yields a calculated pressure drop at the upper surface of the sample of 40 MPa ms⁻¹ in the first 0.15 ms. In turn, the pressure decrease on the upper surface of the sample causes the propagation of a release wave through the sample. The resulting tensile stresses can cause disruption of the porous sample if they exceed its tensile strength. In such a case fragmentation of the

sample might occur, with outburst of gas and particles into the low-pressure tank.

Cored samples of Mount St Helens grey dacite (diameter 17 mm, length 50 mm) similar to cryptodome material (provided by R. Hoblitt) were used in the present experiments. Sample properties were: density, $1,600 \text{ kg m}^{-3}$; total vesicularity (porosity), 34 vol%; open (connected) porosity (by water incorporation), 20 vol%; specific surface area (by BET/nitrogen absorption technique), $0.2 \text{ m}^2 \text{ g}^{-1}$; water content, 0.63 wt% (by Karl-Fischer titration; this volume is within previous estimates of 0.23–0.96 wt% (ref. 11). The volume fraction of phenocrysts is $\sim 30\%$ (ref. 12). The viscosity of samples measured by the parallel-plate viscometry method using a commercial dilatometer (Model TMA 402, Netzsch Gerätebau) yielded extrapolated viscosities of $10^{10.95} \text{ Pas}$ at 885°C , $10^{11.65} \text{ Pas}$ at 825°C and $10^{12.64} \text{ Pas}$ at 750°C . The glass transition temperature ($10^\circ\text{C min}^{-1}$; determined by dilatometry) of the matrix glass is $\sim 800^\circ\text{C}$. Dilatometric measurement of the present sample material indicates that no

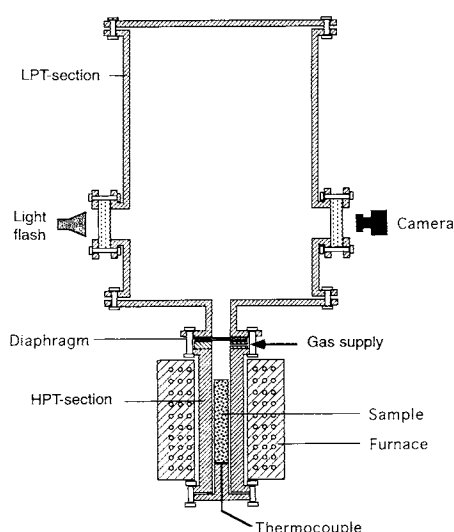


FIG. 1 Schematic illustration of the high-pressure/high-temperature (shock-tube-type) fragmentation device used in this study. The high pressure/temperature section (HPT; internal diameter 20 mm, external diameter 40 mm, length 450 mm) is constructed from heat-treated Nimonic 105 alloy. It was designed to operate under pressures up to 20 MPa, and temperature up to 950°C . Cylindrical lava samples (washed in an ultrasonic bath, dried at 120°C overnight) are placed within thin-walled, tight-fitting Ni tubes. The Ni tube is rigidly connected to a closure plug beneath the sample. A thermocouple is in contact with the sample bottom. There is no significant free space beneath the sample. Samples were heated at a rate of $15^\circ\text{C min}^{-1}$, held at temperature for 1 hour to stabilize the thermal regime, and then slowly pressurized/saturated by argon. The argon is supplied to the high pressure/temperature section through a valve opening into the upper part of this section, and fills the connected pores of the sample as well as the space between the sample and the diaphragm. The distance between the upper surface of the sample and the diaphragm is 210 mm. After diaphragm disruption, the sample experiences rapid decompression. The position of the sample in the high pressure/temperature section corresponds to the hot zone of the section which is heated by an external Kanthal-wound resistance furnace. The low pressure/temperature (LPT) section consists of a 120-mm-long tube with internal diameter 20 mm which vents into a larger steel tank (700 mm diameter, 2,000 mm length). Its volume (0.7 m^3) is much greater than the volume of the high pressure/temperature section ($8 \times 10^{-5} \text{ m}^3$) ensuring a near-constant 'target' pressure in the tank during the expulsion of compressed gas (depressurization). The tank is equipped with windows for observation of fragments during their outburst. A flash light source (flash duration, $0.5 \mu\text{s}$) is used for obtaining still photographs of flying fragments. A dynamic pressure transducer installed near the bursting diaphragm triggers the light flash source through an electronic device after a chosen time delay.

vesiculation occurs at temperatures up to 950°C over several hours (even at atmospheric pressure).

Most of the experiments were performed at an initial pressure differential of about 12 MPa and at temperatures in the range 20 – 885°C . Two sets, each consisting of seven experiments were performed in temperature ranges of 750 – 765°C and 810 – 825°C , respectively. Details of the experimental method are given in Fig. 1 legend.

The experimental conditions described above resulted in the successful fragmentation of Mount St Helens grey dacite. The generated fragments of dacite consisted of angular shards of crystal + vesicular glass (Fig. 2). Examination of fragments by scanning electron microscopy (SEM) did not reveal evidence of any foaming or vesiculation of dacite fragments between rapid decompression and fragmentation (Fig. 2). Frequency curves of the grain-size distribution exhibit simple or bimodal distributions (Fig. 3a). Some of these largest fragments were disk-like in shape (thickness 2–3 mm, diameter 17 mm). The two sets of experiments performed above and below 800°C yielded different grain-size distributions of fragments (Fig. 3b). The median diameters of fragments derived from experiments at 810 – 825°C were in the range -3.1 to -1.7ϕ units (9.9 to 3.3 mm) whereas fragments from experiments at lower temperatures (750 – 765°C) were finer grained (median diameter -2.2 to -1.5ϕ units; 4.6 to 2.8 mm). The higher-temperature experiments generated fragments with sorting coefficients in the range 1.3–2.1; those at lower temperature, in the range 1.3–1.9. Although a number of aspects of the generation and deposition of natural pyroclastics undoubtedly complicate their comparison with the pyroclastics generated in our experiments (for example, steady-state eruption versus single explosion, transport sorting) we note that the textural parameters of fragments obtained in the present experiments are within the limits of fields observed for fallout pyroclastics and pyroclastic flow deposits^{13,14}.

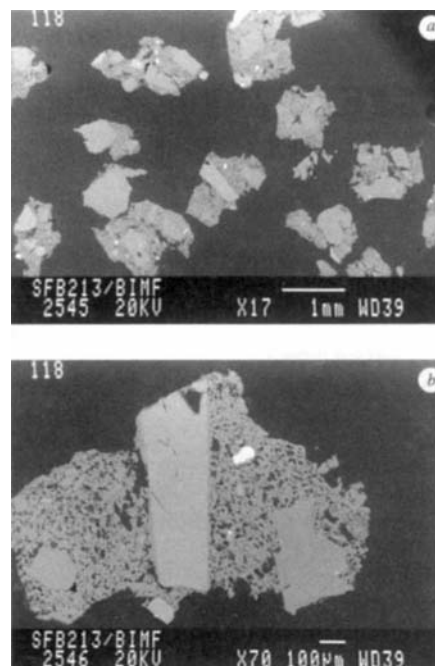


FIG. 2 a, SEM images of fragments of the Mount St Helens grey dacite produced in our experiments by rapid decompression (expt no. 118; $P = 12.6 \text{ MPa}$, $T = 810$ – 825°C). The temperature bounds refer to the ends and the centre of the sample, respectively. Particles consist of crystals and glassy vesicular matrix. They exhibit angular shapes implying brittle fragmentation. b, A detail of a single fragment, illustrating the texture of the experimentally fragmented Mount St Helens grey dacite. The characteristic size is comparable to the phenocryst size. Note the highly irregular shape of the vesicles.

On the basis of the present results we propose the following simple working hypothesis of a mechanism for pyroclast formation (Fig. 4). When the pressure acting at the surface of magma (containing pores with excess gas pressure) suddenly drops, a release (or unloading) wave is initiated which propagates through the vesicular magma column with sound speed c generating dynamic tensile stresses in the magma. If the dynamic tensile stresses generated exceed the dynamic tensile strength of the magma, fracturing and fragmentation occur. Compressed gas in the connected pores expands, opening cracks and accelerating fragments as a gas + fragment outburst. Subparallel crack generation has been observed during rapid depressurization of porous and granular materials^{9,15,16}. Bubbles probably play a special role in fragmentation and ejection by reducing the tensile strength of magma and contributing the potential energy of the compressed gas. This scheme, in which highly viscous magma fragments in response to rapid decompression^{17–19}, is supported by field observations of pyroclast formation^{1,3}.

Magma rheology, together with the properties of the tensile pulse, will dictate the mechanical nature of the magma response (brittle versus ductile)^{20,21}. The effective volume strain rate implied by a decompression rate of 40 MPa ms^{-1} in the first 0.15 ms is $10^{0.6} \text{ s}^{-1}$ (for bulk modulus of magma, $K = 10 \text{ GPa}$). Such a volume strain rate should yield partially unrelaxed or viscoelastic behaviour leading to brittle failure at viscosities as low as three $\log_{10}$ units below those generated by the Maxwell relation for this strain rate, that is, at a critical viscosity of $10^{7.6} \text{ Pa s}$ (ref. 21). It is possible that the increase in fragment size with

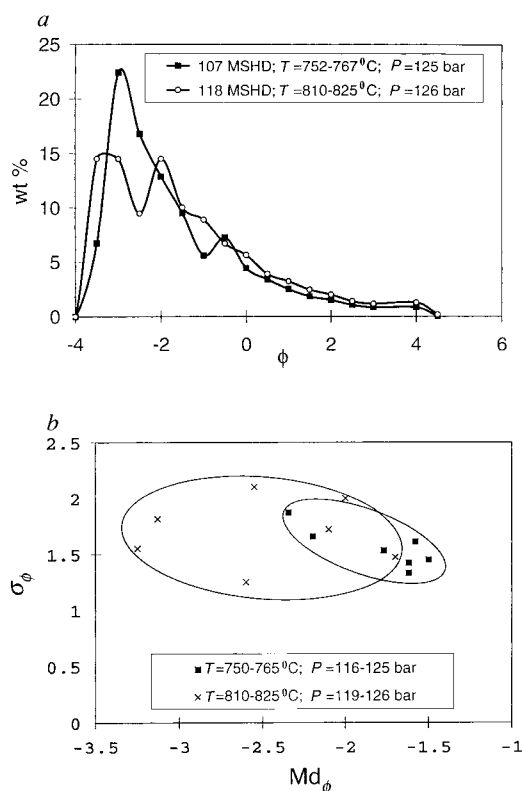


FIG. 3 a, Frequency/grain-size distribution curves (wt% of pyroclasts versus fragment size in ϕ units, where $\phi = -\log_{10} d$ (mm)) for two experiments conducted at similar initial pressure differentials (12.5–12.6 MPa) but contrasting temperatures (expt. no. 107, $T = 752-767^\circ\text{C}$; expt. no. 118, $T = 810-825^\circ\text{C}$). (d , diameter in mm; MSHD, Mt St Helens grey dacite) b, Sorting coefficient ($\sigma_\phi = (\phi_{84} - \phi_{16})/2$, characterizing dispersion¹⁴) versus median diameter (Md_ϕ ; derived from cumulative frequency curves¹⁴) showing the fields of experimental data for two sets of experiments conducted at differing temperatures but similar pressures (see figure key). These overlapping fields illustrate an increase in fragment size with increasing temperature.

FIG. 4 Schematic illustration of a mechanism for the fragmentation and formation of pyroclasts from a highly viscous magma. The initial state of magma before decompression is characterized by a solidified or highly viscous ($\eta > 10^8 \text{ Pa s}$) melt with vesicles (pores) containing gas with pressure in excess of atmospheric ($P_0 > P_a$). The pressure differential between the magma and the atmosphere can be stabilized by a capping layer of rock over a cryptodome magma. At some time after rapid decompression, caused by a landslide involving part of the volcanic edifice, a release wave propagates with sound speed c through the magma. As a result of release wave propagation, tensile stresses occur in certain sections of the magma which generate fractures sub-parallel to the release wave front. The magma is weakened by fractures (cracks) and compressed gas enlarges these cracks during its expansion. Relatively large magma fragments continue to fragment to smaller particles. The mixture of gas and fragments bursts out into the atmosphere.

increasing temperature reflects the growing contribution of viscous (non-brittle) dissipation of energy during decompression as the magma approaches viscoelastic behaviour²⁰. Here we emphasize that variation of the decompression rate at the sample surface influences the volume strain rate and thereby, in principle, the fragmentation process^{20,21}. Similarly, the effective ratio of the initial pore pressure to the strength of the magma may play a controlling role. Future work should include (1) experimental investigation of the dependence of fragmentation character on the initial pressure differential, decompression rate, magma composition and texture (porosity, crystallinity) as well as the resultant rheological and mechanical properties of the magma and (2) detailed comparison of the products of these 'magmatic' experiments with hydromagmatic experimental products^{22,23} and natural products of both types of eruption.

The present observation that pyroclast formation and outburst occurs even at very low temperatures should be incorporated into the evaluation of volcanic hazards. Relatively cool magmas, subjected to rapid unloading events, may be very hazardous. $\square$

Received 11 July 1995; accepted 6 February 1996.

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ACKNOWLEDGEMENTS. We thank H. Grönig, M. Lennarts, P. Neuwald and H. Keppler for experimental advice, R. Stevenson and S. Webb for assistance with rheological measurements, G. Herrmannsdörfer for technical assistance, K. Klasinski and R. Weigel for development of electronic and computer interfacing, and H. Schulze for sample preparation. This work was supported by the Alexander von Humboldt Foundation, the European Commission, the Deutsche Forschungsgemeinschaft and by the Bayerisches Geoinstitut.

A comprehensive genetic map of the mouse genome

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THE availability of dense genetic linkage maps of mammalian genomes makes feasible a wide range of studies, including positional cloning of monogenic traits, genetic dissection of polygenic traits, construction of genome-wide physical maps, rapid marker-assisted construction of congenic strains, and evolutionary comparisons^{1,2}. We have been engaged for the past five years in a concerted effort to produce a dense genetic map of the laboratory mouse³⁻⁶. Here we present the final report of this project. The map contains 7,377 genetic markers, consisting of 6,580 highly informative simple sequence length polymorphisms integrated with 797 restriction fragment length polymorphisms in mouse genes. The average spacing between markers is about 0.2 centimorgans or 400 kilobases.

To construct a simple sequence length polymorphism (SSLP) map, we identified more than 9,000 sequences from random genomic clones and public databases containing simple sequence repeats (mostly, (CA)_n-repeats), designed polymerase chain reaction (PCR) primers flanking the repeat, and tested each for polymorphism by measuring the allele sizes in 12 inbred mouse strains. Of the successful PCR assays, we genotyped the 90% of loci that revealed different alleles between the OB and CAST strains in an (OB × CAST) F₂ intercross with 46 progeny. These data were assembled into a map by performing genetic linkage analysis with the MAPMAKER computer package^{7,8}.

A total of 6,336 SSLP loci were scored in the F₂ intercross, with 6,111 derived from anonymous sequence and 225 from known genes (Table 1). Of these, 5,905 were scored as codominant markers and 431 as dominant markers (because the pattern of one allele obscured the other). The map provides dense coverage of all 20 mouse chromosomes, with a total genetic length of 1,361 centimorgans (cM). Because the cross involves 92 meioses, the mean spacing between crossovers is 1.1 cM and thus loci can be mapped to 'bins' of this average size. The map has 1,001 occupied bins (Table 3(a)), with an average of 6.3 markers per bin and an average spacing of 1.36 cM between consecutive bins.

We next sought to integrate the map of largely anonymous SSLPs with the locations of known genes, because this information can suggest candidates for the genes underlying mouse mutations. We analysed a (B6 × SPRET) backcross that has been extensively used for restriction fragment length polymorphism (RFLP) mapping⁹⁻¹¹. The backcross has been genotyped for 797 RFLPs. To integrate the maps, we genotyped 1,245 SSLPs from our map in 46 progeny from the SPRET backcross, providing a common reference point approximately every 1.1 cM. We also genotyped 244 additional SSLPs that were not polymorphic—and thus could not be mapped—in the (OB × CAST) intercross, but were polymorphic in the (B6 × SPRET) backcross. The SPRET cross was thus scored for a total of 1,543 SSLPs and 797 RFLPs.

The final map with 7,377 loci is shown in Fig. 1, with the SSLP map on the right and the integration with the RFLP map on the left. A full description of the markers—including primer sequences, locus sequence, genotypes in each cross, and allele

TABLE 1 Genetic markers, genetic length and polymorphism* by chromosome

Chromosome	No. of markers	No. of random markers	No. from GENBANK	'Consensus' genetic length†	Observed genetic length‡	Polymorphism among lab strains (%)§	Lab strains versus SPR or CAST (%)
1	511	494	17	98	109.9	57	92
2	507	491	16	107	95.7	49	94
3	343	332	11	100	67.5	51	95
4	350	342	8	81	74.2	51	93
5	402	391	11	93	82.9	48	95
6	368	349	19	74	59.1	46	94
7	357	341	16	89	59.8	48	94
8	350	345	5	81	72.0	44	94
9	336	318	18	70	62.9	52	95
10	293	286	7	78	73.0	35	96
11	350	326	24	78	82.0	53	94
12	278	268	10	68	61.5	50	94
13	303	296	7	72	60.2	48	95
14	259	246	13	53	65.6	49	94
15	264	257	7	62	62.2	51	94
16	215	214	1	59	51.0	43	94
17	255	239	16	53	51.0	56	93
18	231	226	5	57	39.7	53	95
19	134	131	3	42	57.2	52	93
X	230	219	11	88	73.5	33	95
Total	6,336	6,111	225	1,503	1,360.9¶	48	94

* Polymorphism survey was based on visual comparisons of fragments across large acrylamide gels and was thus subject to mobility differences among lanes. To assess the accuracy of data in our database, 3,000 individual pairwise comparisons were repeated. Some 6% of reported polymorphic pairs turn out to be monomorphic upon careful comparison, while 4% of reported monomorphic pairs turn out to be polymorphic. The data are thus accurate enough to allow selection of markers for crosses, but geneticists wishing to know every polymorphic marker in a narrow region (for fine-structure genetic mapping and positional cloning, for example) are advised to recheck each locus.

† Based on 'consensus' genetic map in Encyclopedia of the Mouse Genome, <http://www.informatics.jax.org/encyclo.html> (1993).

‡ Distance between most proximal and most distal markers in the map reported here.

§ Pairwise comparisons of OB, B6, DBA, A, C3H, BALB, AKR, NON, NOD and LP.

|| Standard error of the mean for each chromosome depends on number of markers studied, but is < 1% in all cases.

¶ Distance is shorter than in previously published versions of this map (ref. 6) because final error checking reduced the number of apparent crossovers.

TABLE 2 Distribution of random markers based on cytogenetic length of chromosomes

		Based on cytogenetic length*		
Chromosome	No. of random markers†	Percentage of total length	Expected number of markers‡	Z-score§
Autosomes only				
1	494	7.68 ± .15	452.7 ± 22.4	1.84
2	491	7.42 ± .15	437.0 ± 21.9	2.47
3	332	6.39 ± .13	376.7 ± 20.2	-2.20
4	342	6.29 ± .13	360.4 ± 20.0	-1.41
5	391	6.06 ± .12	356.2 ± 19.7	1.73
6	349	5.90 ± .12	347.7 ± 19.4	0.07
7	341	5.54 ± .11	326.4 ± 18.7	0.79
8	345	5.30 ± .11	312.5 ± 18.3	1.78
9	318	5.11 ± .10	301.2 ± 17.9	0.94
10	286	5.06 ± .10	298.1 ± 17.8	-0.67
11	326	5.04 ± .10	296.8 ± 17.8	1.65
12	268	5.21 ± .10	306.9 ± 18.1	-2.14
13	293	4.67 ± .09	275.4 ± 17.1	1.21
14	246	4.76 ± .10	280.5 ± 17.3	-1.99
15	257	4.32 ± .09	254.7 ± 16.4	0.15
16	214	4.07 ± .08	239.6 ± 15.9	-1.60
17	239	4.12 ± .08	242.7 ± 16.0	-0.22
18	226	4.14 ± .08	244.0 ± 16.0	-1.11
19	131	2.91 ± .06	171.7 ± 13.4	-3.04
Total	5,892	100.0	5,892.0	
Autosomes versus X chromosome				
Autosomes	5,892	93.76 ± .12	5,729.7 ± 20.4	7.96
X	219	6.24 ± .12	381.3 ± 20.4	-7.96
Total	6,111	100.0	6,111.0	

* Cytogenetic length taken from previous measurements¹⁹. Standard error of the mean was calculated directly from the raw data on chromosome measurements, generously provided by E. Evans.

† Only random markers are considered to avoid biases in chromosomal distribution of known genes.

‡ Mean ± standard deviation. Standard deviation in number of markers expected combines both standard error in the measurement of chromosome length and sampling error given to the total number of loci examined. Uncertainty in the precise length of chromosomes was not included in previous analyses⁶, owing to its small magnitude, but it becomes relevant as the number of loci increases and sampling error correspondingly decreases. For comparison of autosomes to X chromosome, the expectation reflects the fact that 5% of the random markers were derived from male DNA (thus underrepresenting the X chromosome by a factor of two) and 95% from female DNA.

§ Z-score = (observed - expected)/standard deviation. For the autosomes, all of the Z-scores are significant at the $P = 0.05$ level after Bonferroni correction for multiple testing. For the comparison of autosomes to X chromosome, the Z-score is significant at $P < 10^{-14}$.

sizes in the characterized strains—would require over 500 pages of this journal. The complete information is available electronically on the WorldWide Web (see Fig. 1 legend).

The maps constructed in the CAST intercross and SPRET backcross maps have similar lengths (1,361 and 1,385 cM respectively), despite the fact that the intercross reflects sex-averaged recombination rates and the backcross reflects female recombination rates (because heterozygous mothers were used). Because there is typically about 80% more recombination in females than males, the SPRET backcross map might be expected to be about 40% longer. That it is not probably reflects recombinational suppression owing to structural heterogeneity (inasmuch as the laboratory mouse is evolutionarily twice as distant from SPRET as from CAST).

The SSLP map constructed in the cross was subjected to rigorous quality control and quality assessment^{3,8}. All obligate double crossovers were identified and rechecked. The final data set contained no obligate double crossovers involving markers separated by less than 21 cM, indicating strong crossover interference in the mouse. (In the absence of interference, about 100 such events would be expected.) We also filled in any missing genotypes that could alter the position of a locus (by virtue of being adjacent to the site of a crossover). Despite our best efforts, some errors surely remain: in particular, an incorrect genotype adjacent to the site of a crossover would not necessarily produce a double crossover, and could shift a locus by 1.1 cM. Each chromosome is thus likely to contain a handful of loci that are slightly misplaced. The SSLPs used for integration with the SPRET backcross provided a different assessment of accuracy. We checked whether these 1,245 loci mapped to the same location in both crosses. There were ten apparent discrepancies. In five cases (*D5Mit198*, *D7Mit173*, *D9Mit132*, *D9Mit150* and *D19Mit61*), the loci were found to reproducibly amplify polymorphic fragments at different chromosomal locations in the two crosses. This probably occurs because strain variation creates an alternative

target for amplification, although the possibility that CAST and SPRET differ by small insertional translocations cannot be excluded. In remaining five cases, the results from the CAST cross were found not to be reproducible. These probably arose from laboratory errors that unfortunately cannot be identified in retrospect. These five loci were removed from the map. Based on the frequency (5 of 1,245), we would expect that 20 further erroneous loci remain, which corresponds to about one per chromosome.

We used several criteria to analyse the genomic distribution of loci. The spacing between SSLPs agrees reasonably well with expectation under a random distribution, although some deviation from randomness can be detected. The relative positions of markers and crossovers can be inferred completely in an experimental cross, and the entire data set can be reduced to a string of the form 'mmccmmmmccmcmcm...', with each m and c denoting the occurrence of a marker or a crossover, respectively. The hypothesis that markers are randomly distributed with respect to crossovers can be tested by comparing the observed clustering of consecutive markers and crossovers to that expected for tossing a biased coin with the probability of a marker being $p_m = M/(M + C)$, where M is the number of markers and C the number of crossovers⁶. There is some statistically significant evidence of clustering by this test (Table 3). The map contains an interval with eight consecutive crossovers (on chromosome 19) and a block of 54 recombinationally inseparable markers (on chromosome 2); the probability of such clusters of crossovers and markers occurring at random somewhere in the map is 0.5% and 3.4%, respectively. More generally, the frequency of both large and small clusters slightly exceeds expectation. Nonetheless, the distribution is not far from random expectation, at least at the level of resolution provided by the meioses studied here.

The chromosomal distribution of SSLPs among the autosomes agrees well with expectation under the assumption that loci are uniformly distributed with respect to cytogenetic length.

(Chromosome 19 shows a slight deficit, which is not statistically significant after correction for multiple testing; it may reflect the unusually large proportion of heterochromatin on this chromosome.) In contrast, chromosome X shows a clear deficit, with only about 57% as many as expected (Table 2). This phenomenon appears to be general in mammalian genomes, as we have also found a similar deficit in an SSLP map of the rat¹² (62% of expectation), and Weissenbach and colleagues report a slightly less pronounced deficit in the human genome¹³ (75% of expectation). In principle, the deficit of SSLPs on chromosome X could occur if (CA)_n-repeats were either less frequent on chromosome X, or were equally frequent but less polymorphic. The latter hypothesis would predict that the deficit of polymorphic loci on chromosome X would be offset by a great excess of non-polymorphic repeats. Of the SSLPs monomorphic between OB and CAST, 37% would have to lie to chromosome X to explain the observed data. We determined the chromosomal location of > 100 monomorphic loci (by genetic mapping for those that were polymorphic between B6 and SPRET and by somatic cell hybrid mapping for those that were not), but we found no significant excess on chromosome X. Accordingly, the deficit appears to be primarily due to an actual shortage of (CA)_n-repeats on chromosome X.

The SSLPs show a polymorphism rate of about 50% among inbred laboratory strains surveyed and about 95% between laboratory strains and CAST or SPR (Table 1). The pairwise polymorphism rates among the 12 strains surveyed have not changed significantly from our previous report⁶ and are not presented here. Interestingly, the distribution of polymorphism across the genome is not uniform¹¹. The average polymorphism rate among the *Mus musculus* strains surveyed was just under 50%, but two chromosomes showed substantially lower polymorphism rates: chromosome X at 33%, and chromosome 10 at 35% (Table 1). Decreased polymorphism could reflect recent selection for specific ancestral chromosomes. For the X chromosome, it could also reflect a different mutation rate (inasmuch as each chromosome X resides in males only two-thirds as often each autosome, and most mutations are thought to occur in male germline) or different population genetic forces (with hemizygosity affecting selection and effective population size).

Our mouse genetic-mapping project is now at its conclusion. Although more SSLPs remain to be found (newly

TABLE 3 Clusters of consecutive crossovers and markers

(a) Number of crossovers between consecutive random markers*

No. of crossovers per interval	Observed		Expected†		P(longest run $\geq n$) (%)‡
	No.	(percentage)	No.	(percentage)	
0	5,095	(83.85)	5,035.5 $\pm$ 29.6	(82.59)	
1	784	(12.90)	876.7 $\pm$ 27.4	(14.38)	100.0
2	151	(2.49)	152.6 $\pm$ 12.2	(2.50)	100.0
3	27	(0.44)	26.6 $\pm$ 5.1	(0.44)	100.0
4	14	(0.23)	4.6 $\pm$ 2.2	(0.08)	99.6
5	4	(0.07)	0.8 $\pm$ 0.9	(0.01)	62.2
6	0	(0.00)	0.1 $\pm$ 0.4	(< 0.01)	15.6
7	0	(0.00)	0.0 $\pm$ 0.2	(< 0.01)	2.9
8	1	(0.02)	0.0 $\pm$ 0.1	(< 0.01)	0.5
Total	6,076				

(b) Random markers occurring between consecutive crossovers‡

No. of markers per block	Observed		Expected§		P(longest run $\geq n$) (%)§
	No.	(percentage)	Number	(percentage)	
0	288	(22.3)	227.9 $\pm$ 13.7	(17.4)	100.0
1	208	(16.1)	188.2 $\pm$ 12.7	(14.4)	100.0
2	126	(9.8)	155.5 $\pm$ 11.7	(11.9)	100.0
3	111	(8.6)	128.4 $\pm$ 10.8	(9.8)	100.0
4	84	(6.5)	106.0 $\pm$ 9.9	(8.1)	100.0
5	73	(5.7)	87.6 $\pm$ 9.0	(6.7)	100.0
6	62	(4.8)	72.3 $\pm$ 8.3	(5.5)	100.0
7	51	(4.0)	59.7 $\pm$ 7.6	(4.6)	100.0
8	36	(2.8)	49.3 $\pm$ 6.9	(3.8)	100.0
9	38	(2.9)	40.7 $\pm$ 6.3	(3.1)	100.0
10	32	(2.5)	33.7 $\pm$ 5.7	(2.6)	100.0
11	37	(2.9)	27.8 $\pm$ 5.2	(2.1)	100.0
12	19	(1.5)	23.0 $\pm$ 4.7	(1.8)	100.0
13	28	(2.2)	19.0 $\pm$ 4.3	(1.4)	100.0
14	18	(1.4)	15.7 $\pm$ 3.9	(1.2)	100.0
15	7	(0.5)	12.9 $\pm$ 3.6	(1.0)	100.0
16	12	(0.9)	10.7 $\pm$ 3.3	(0.8)	100.0
17	5	(0.4)	8.8 $\pm$ 3.0	(0.7)	100.0
18	5	(0.4)	7.3 $\pm$ 2.7	(0.6)	100.0
19	6	(0.5)	6.0 $\pm$ 2.4	(0.5)	100.0
20	10	(0.8)	5.0 $\pm$ 2.2	(0.4)	100.0
21	3	(0.2)	4.1 $\pm$ 2.0	(0.3)	100.0
22	5	(0.4)	3.4 $\pm$ 1.8	(0.3)	100.0
23	7	(0.5)	2.8 $\pm$ 1.7	(0.2)	100.0
24	4	(0.3)	2.3 $\pm$ 1.5	(0.2)	100.0
25	0	(0.0)	1.9 $\pm$ 1.4	(0.1)	100.0
26	5	(0.4)	1.6 $\pm$ 1.3	(0.1)	99.9
27	1	(0.1)	1.3 $\pm$ 1.1	(0.1)	99.8
28	1	(0.1)	1.1 $\pm$ 1.0	(0.1)	99.3
29	0	(0.0)	0.9 $\pm$ 0.9	(0.1)	98.4
30	1	(0.1)	0.7 $\pm$ 0.9	(0.1)	96.7
31	1	(0.1)	0.6 $\pm$ 0.8	(< 0.1)	94.0
32	0	(0.0)	0.5 $\pm$ 0.7	(< 0.1)	90.3
33	0	(0.0)	0.4 $\pm$ 0.6	(< 0.1)	85.4
34	1	(0.1)	0.3 $\pm$ 0.6	(< 0.1)	79.6
35	1	(0.1)	0.3 $\pm$ 0.5	(< 0.1)	73.1
38	1	(0.1)	0.2 $\pm$ 0.4	(< 0.1)	52.2
40	1	(0.1)	0.1 $\pm$ 0.3	(< 0.1)	39.6
54	1	(0.1)	< 0.1 $\pm$ 0.1	(< 0.1)	3.4
Total	1,289				

* The intervals with ≥ 1 crossover represent the 981 gaps between consecutive bins of recombinationally inseparable markers. Only random markers are considered to avoid biases in distribution of known genes.

† The probability of the longest run is calculated in ref. 6. Briefly, if a coin with heads probability P is tossed n times, the length R_n of the longest head run has expected value $\mu = \log_{1/P}[(n-1)(1-P) + 1]$ and the distribution of R_n is given approximately by $\text{Prob}(R_n - \mu > t) = 1 - \exp(-P^t)$. In this case, $p = 0.17$.

‡ The blocks with ≥ 1 marker represent the 1,001 bins of recombinationally separable markers. Only random markers are considered to avoid biases in distribution of known genes.

§ The probability of the longest head run is calculated with $p = 0.83$.

isolated repeats show < 10% overlap with our current set), we have reached the point of diminishing returns. The map covers the entire mouse genome, with the markers being sufficiently abundant, polymorphic and stable to allow the mapping of monogenic or polygenic traits in virtually any mouse cross of interest^{5,8}. Moreover, the markers are sufficiently dense to facilitate positional cloning of most mouse mutations. With > 90% of the mouse genome being within 750 kb of a marker, and current mouse yeast artificial chromosome (YAC) libraries^{14,15} having a mean insert size > 750 kb, the map affords ready access to the vast majority of the genome with little need for chromosomal walking, and provides a preliminary scaffold for constructing a genome-wide physical map¹⁶.

The map also provides a common framework for the mapping of mutations and cloned genes. In addition to our integration with the Frederick cross, the SSLP map is being used as a framework for other mapping crosses, including public resources at the Jackson Laboratory¹⁷ and the European Collaborative Interspecific Backcross (EUCIB)¹⁸. The EUCIB project (<http://www.hgmp.mrc.ac.uk/MBx/MBxHomepage.html>) is rescoring our SSLP markers in a cross with 1,000 meioses, which should yield finer resolution of order and correct remaining errors.

Together with the final report on the human genetic map¹³, this paper marks the close of the first phase of the Human Genome

Project: the construction of dense genetic maps of mouse and man. □

Received 23 October 1995; accepted 19 February 1996.

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ACKNOWLEDGEMENTS. We thank L. Wangchuk, D. Tsering, G. Farino and K. Norbu for technical assistance; D. Gilbert and L. Maltais for help in ascertaining official nomenclature for gene loci; and Research Genetics Inc. for making SSLP primers available to the community. This work was supported in part by a grant from the National Center for Human Genome Research (to E.S.L.). L.K. was supported by a Special Emphasis Research Career Award from the National Center for Human Genome Research.

A comprehensive genetic map of the human genome based on 5,264 microsatellites

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THE great increase in successful linkage studies in a number of higher eukaryotes during recent years has essentially resulted from major improvements in reference genetic linkage maps^{1–6}, which at present consist of short tandem repeat polymorphisms of simple sequences or microsatellites^{7,8}. We report here the last version of the Généthon human linkage map⁶. This map consists of 5,264 short tandem (AC/TG)_n repeat polymorphisms with a mean heterozygosity of 70%. The map spans a sex-averaged genetic distance of 3,699 cM and comprises 2,335 positions, of which 2,032 could be ordered with an odds ratio of at least 1,000:1 against alternative orders. The average interval size is 1.6 cM; 59% of the map is covered by intervals of 2 cM at most and 1% remains in intervals above 10 cM.

Microsatellite markers were obtained as described previously^{5,6}. A heterozygosity above 0.5 was observed for 93% of the markers and above 0.7 for 58%. These values remain very close to those of our previous version⁶. Average heterozygosity per chromosome varied from 0.65 (chromosome X) to 0.73 (chromosome 19), with a mean value of 0.70 for the entire collection of markers (Table 1). Database sequence comparisons and searches detected matches of AFM (Association Française contre les Myopathies) markers with 19 genes and 74 anonymous markers.

Genotyping of the microsatellite markers was performed as described previously on the same eight CEPH (Centre d'Etudes du Polymorphisme Humaine) families (20 for the X chromosome), which comprised a total of 134 individuals and 186 meioses^{5,6} (304 individuals and 291 meioses for the X chromosome). Genotypes were submitted to the same error-checking procedures as reported earlier⁶. These procedures consisted of (1) a reinvestigation of families with abnormally elevated recombination frequencies between pairs of markers, and (2) correction or elimination of all double recombinant genotypes of markers placed in short linkage intervals. Such apparent double recombinations probably result from mutation events that converted an allele of one individual into the other allele. A more detailed analysis of double-recombination events and mutations in microsatellites is in preparation.

Map construction was done in a stepwise manner with multiple controls at each step. The total length of this map as evaluated from the CILINK algorithm⁹ is 3,699 cM (Table 1). This is almost identical in length to our previous version, despite the addition of new terminal markers that extend the 93/94 chromosome maps by 145 cM (4%). The absence of increase in length probably results from a very thorough error-checking process and from elimination of apparent double-recombinant genotypes. The 5,264 markers are distributed in 2,335 positions (Fig. 1), 2,032 of which are ordered with odds ratios against alternative orders of at least 1,000:1. The mean interval size is 1.6 cM. The fraction of the map in intervals above 10 cM represents only 1 per cent of the total linkage distance and consists of 3 intervals spanning 11 cM. Fifty-nine per cent of the map is covered by intervals of 2 cM at most, and 92 per cent by intervals of 5 cM at most. Markers from the CEPH and CHLC databases have been integrated into this map as shown in Fig. 2, which presents the map of chromosome 22 as an example. Detailed information, including integrated maps of all chromosomes, a list of markers, their primer sequences, heterozygosity, number and size-range of alleles observed in the 8 (or 20) genotyped CEPH families, sex-specific distances, and mutations, will be presented in an extended reprint available on request and on an electronic server (<http://www.genethon.fr>).

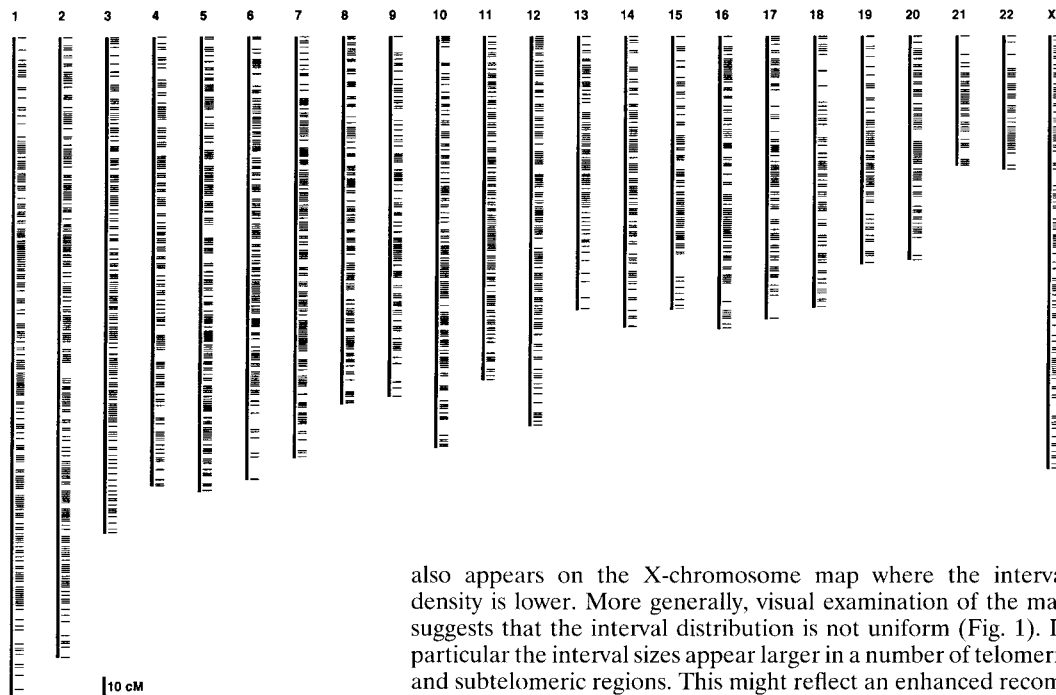
The total sex-specific lengths of autosomes estimated by CILINK⁹ show only slight variations when compared to the lengths of the previous map⁶. The length excess observed for the female map is comparable to other published maps. This excess

TABLE 1 Quantitative characteristics of maps and markers by chromosome

Chromosome	Physical length* (Mb)	Number of markers mapped	Markers per Mb	Genetic length (cM)†			Markers per cM	Mean heterozygosity‡	Ordered 1,000:1§
				Female	Male	Sex-average			
1	263	461	1.75	358.2	220.3	292.7	1.57	0.71	142
2	255	452	1.77	324.8	210.6	277.0	1.63	0.71	160
3	214	353	1.65	269.3	182.6	233.0	1.51	0.71	199
4	203	280	1.38	270.7	157.2	212.2	1.32	0.69	103
5	194	312	1.61	242.1	147.2	197.6	1.57	0.70	117
6	183	311	1.70	265.0	135.2	201.1	1.54	0.71	116
7	171	272	1.59	187.2	178.1	184.0	1.48	0.72	116
8	155	249	1.61	221.0	113.1	166.4	1.50	0.70	93
9	145	189	1.30	194.5	138.5	166.5	1.13	0.71	67
10	144	281	1.95	209.7	146.1	181.7	1.55	0.70	101
11	144	273	1.90	180.0	121.9	156.1	1.75	0.70	104
12	143	249	1.74	211.8	126.2	169.1	1.47	0.71	95
13q	98	164	1.67	132.3	97.2	117.5	1.40	0.70	61
14q	93	162	1.74	154.4	103.6	128.6	1.26	0.72	70
15q	89	145	1.63	131.4	91.7	110.2	1.36	0.70	52
16	98	180	1.84	169.1	98.5	130.8	1.38	0.71	75
17	92	186	2.02	145.4	104.0	128.7	1.44	0.70	68
18	85	136	1.60	151.3	92.7	123.8	1.10	0.70	50
19	67	121	1.81	115.0	98.0	109.9	1.10	0.73	51
20	72	144	2.00	120.3	73.3	96.5	1.49	0.70	52
21q	39	61	1.56	70.6	46.8	59.6	1.02	0.70	27
22q	43	67	1.56	74.7	46.9	58.1	1.15	0.71	32
X	164	216	1.32	198.1		198.1	1.09	0.65	81
Genome	3,154	5,264	1.67	4,396.9	2,729.7	3,699.2	1.38	0.70	2,032

* Physical lengths are from ref. 18. † Genetic lengths were determined using the CILINK program of the LINKAGE package⁹. ‡ Heterozygosities were determined using genotypes of 56 autosomes (or 42 X chromosomes) from unrelated caucasoid individuals (CEPH grandparents of families 884, 1331, 1332, 1347, 1362 and 1416 and parents of families 102 and 1413). § 1,000:1 odds order was determined as for Fig. 1.

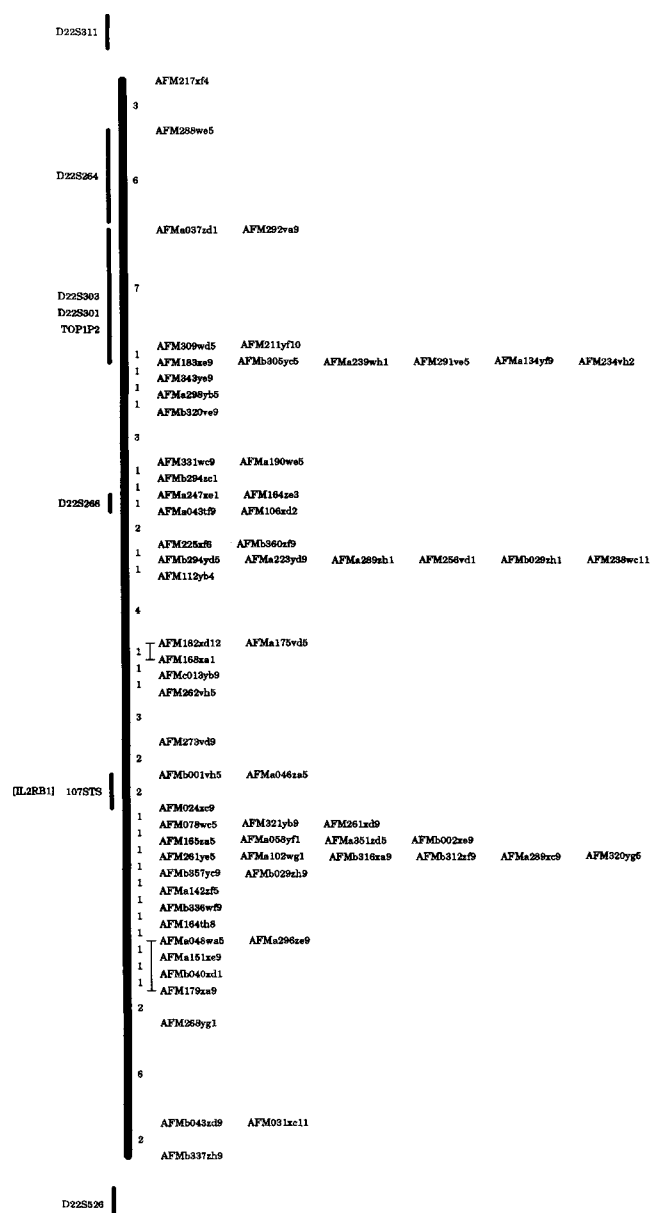
FIG. 1 Genetic linkage map of the 2,335 positions defined by 5,264 markers. The vertical bar delimits the length covered by the map as computed by the GMS algorithm¹⁹ and represents sex-averaged genetic distances in cM. Horizontal marks represent map positions of AFM microsatellite markers. All distance values between positions were rounded to integer values and distances between 0 and 1 cM were rounded to 1 cM. Slight differences may appear between distances evaluated using GMS (this figure) and CILINK displayed in Table 1. This reflects the fact that clusters of non-recombinant markers were processed in a different manner by each program. Maps were constructed using the automated map construction algorithm MultiMap²⁰ based on CRIMAP²¹. The order proposed by MultiMap (primary map) was further submitted to another algorithm, GMS, based on the LINKAGE package⁹. For markers not ordered with the support of 1,000:1 odds on the primary map, the most likely order was chosen. Markers for which there were two equally probable and



'most likely' positions usually did not recombine with another marker situated between those two positions. Absence of recombination between such markers was verified and they were submitted as non-recombinants for the GMS computation. The order of loci proposed by the GMS algorithm (secondary map) was further checked for double recombinants. Given the high density of markers, these double recombinant genotypes were very unlikely and therefore subjected to rescoring. Among a total of 874 double recombinant genotypes rescored, 71% were reassessed. A second genotyping experiment was carried out for suspicious genotypes. This procedure was used until the latest secondary order no longer revealed new double recombinant genotypes. The GMS algorithm usually computes sets of 15 to 20 markers from segments of a chromosome. For the final GMS computation, these segments were defined manually to maximize the number of positions that could be ordered with 1,000:1 odds. A total of 128 double recombinants could not be corrected. These apparent double recombinations occurred in very small intervals surrounded by several markers of the alternative phase. Moving them by a few cM did not solve the problem or resulted in an increased number of alternative double recombinant genotypes on other haplotypes. Because such apparent double recombination events are most probably mutations, they were eliminated from the dataset for the final map calculations.

also appears on the X-chromosome map where the interval density is lower. More generally, visual examination of the map suggests that the interval distribution is not uniform (Fig. 1). In particular the interval sizes appear larger in a number of telomeric and subtelomeric regions. This might reflect an enhanced recombination frequency in such regions. To study the distribution of genetic markers throughout the human genome, we designed a simulation model that could account for variations resulting from marker identification and typing processes. On average, the observed numbers are close to the simulation using a density of two highly informative $(AC)_n$ microsatellites per cM without significant bias in the observed marker distribution (results not shown).

The physical sizes of the largest gaps were estimated by measuring the number of radiation-induced breaks in a panel of whole-genome radiation hybrids developed recently¹⁰. Assuming that breakage distances of radiation hybrid maps are representative of physical distances, 3 out of 4 large genetic intervals tested are notably smaller than expected from the linkage distance (results not shown). This suggests that a number of the gaps remaining on the genetic map correspond to regions with



enhanced genetic recombination rather than to segments devoid of (AC)_n microsatellites. However, as seen for one of the gaps analysed, some intervals may represent actual physical gaps. Similarly, clusters of non-recombining markers can be separated by distances estimated to be of the order of several megabases.

FIG. 2 Integrated genetic linkage map of chromosome 22. All chromosome maps are presented in the extended reprint, as illustrated in this example for chromosome 22. The leftmost column gives names of genes or non-AFM anonymous loci from the CEPH/CHLC databases. These loci were positioned by the MultiMap algorithm at 1,000:1 odds with respect to a framework of AFM markers. Gene names framed by brackets ([IL2RB1]) indicate genes for which a microsatellite was specifically developed as a genetic marker. These gene markers were genotyped, error-checked and positioned using the same criteria as for AFM markers. The vertical lines on the left indicate the intervals in which genes or non-AFM anonymous loci from the CEPH/CHLC databases could be placed by MultiMap at odds >1,000:1. The thick vertical bar delimits the length covered by the map as computed by the GMS algorithm. Some loci were placed at 1,000:1 odds outside the AFM framework; they are depicted on the top and/or bottom of the maps. Because genotypes of these loci were not submitted to the same quality control procedures as AFM markers, only relative positions, but not distances are presented, that is, their distance from other markers and the length of the interval corresponding to their position is arbitrary. AFM markers are displayed in the right part of the map from top to bottom according to the GMS defined order and distances. Markers appearing on the same line are markers with no recombinations in the informative subset of the 8 CEPH families tested. The order of AFM markers on a line is arbitrary. Numbers to the right of the thick bar between consecutive AFM markers correspond to sex-averaged genetic distances in cM evaluated by GMS between consecutive AFM markers. All values comprised between 0 and 1 cM were rounded to 1 cM. The vertical segments in front of the name of AFM markers indicate groups of markers for which the order could not be resolved with odds >1,000:1.

METHODS. Integration of other loci: sequence comparisons and searches in databases detected matches of AFM markers with 19 genes and 74 anonymous markers. These genes or loci, which had been independently assigned to the same chromosomes (except in one instance), could thus be precisely positioned on the present maps. We have attempted to include markers from the CEPH and CHLC databases in our maps. A framework map based on 1,844 AFM markers ordered at 1,000:1 odds was established using the MultiMap algorithm. An additional 186 non-AFM markers could be positioned at 1,000:1 odds on this predefined MultiMap framework and are indicated on the maps.

The highly informative microsatellites enabled us to order more than 2,000 markers with an odds ratio of 1,000:1 with the genotyping of only eight reference CEPH families representing a total of 186 informative meioses at best. But we gave priority to marker density rather than to degree of resolution. With this dense map and the additional genetic mapping resources available^{2,11-15}, linkage mapping of monogenic diseases can be readily carried out to the centimorgan level in most instances. In addition, the availability of numerous highly informative markers will also serve to define regions of linkage disequilibrium in which a founder haplotype can be reconstituted in genetically isolated populations. The genome-wide searches for loci involved in complex diseases will also considerably benefit from this new resource. In addition, this map provides the scaffold for the construction of physical maps based on overlapping sets of yeast artificial chromosomes that have been recently published^{16,17}. □

Received 6 October 1995; accepted 8 January 1996.

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ACKNOWLEDGEMENTS. This work was initiated at CEPH and results from discussions with D. Cohen. It was supported by the Association Française contre les Myopathies, the Groupement de Recherches et d'Études sur les Génomés and European Union (Biomed1). We acknowledge the technical and clerical contribution of L. Baron, N. Becuwe, M. Besnard-Gonnet, I. Bordelais, C. Caloustian, C. Cruaud, M. Dubois, C. Dumont, C. Dupraz-Ramel, E. Ernst, K. Fonsat, M. François, J. L. Mangua, C. Marquette, M. Mauge, E. M. Bene, M. Meugnier, D. Muselet, S. Nguyen, S. Pezard, M. Tranchant, N. Surin, N. Vega, E. Wunderle and V. Wunderle. The group of summer students substantially contributed to the data collection and error checking. We thank the informatics team of Génethon, particularly X. Benigni, L. Bougueleret, C. Discala, J.-M. Froussard, R. Gavrei, P. Gesnouin, S. Pook, P. Rodriguez-Tomé, L. Sainte-Marthe, C. Scarpelli and G. Vaysseix. We also thank A. de Sario and G. Bernardi for the DNA isochore fractionation, S. Cure for help in writing the manuscript, and G. Peirano for continuous devotion to this project.

Non-mutualistic yucca moths and their evolutionary consequences

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INTERSPECIFIC mutualisms are regarded as having evolved from antagonistic or commensalistic interactions, with most mutualisms remaining facultative but some having coevolved into obligate reciprocal dependency¹⁻⁴. Underlying mutualism is an intrinsic conflict between the parties, in that each is under selection for increased exploitation of the other³⁻⁷. Theoretical models suggest that this conflict is a source of evolutionary instability, and that evolution of 'cheating' by one party may lead to reciprocal extinction^{4,6,7}. Here we present phylogenetic evidence for reversal of an obligate mutualism: within the yucca moth complex, distinct cheater species derived from obligate pollinators inflict a heavy cost on their yucca hosts by laying their eggs but not pollinating the yucca. Phylogenetic data show the cheaters to have existed for a long time. Coexisting pollinators and cheaters are not sister taxa, supporting predictions that evolution of cheating within a single pollinator is evolutionarily unstable. Several lines of evidence support a hypothesis that host shifts preceded the reversal of obligate mutualism. Host or partner shifts is a mechanism that can provide a route of evolutionary escape among obligate mutualists in general.

The obligate mutualism between yuccas and yucca moths is one of the classical cases of interspecific cooperation^{1,4,5,8}. The female moth uses unique tentacular mouthparts to collect and transport pollen among yucca flowers. She oviposits into ovaries, and then actively pollinates the flower. Her progeny feed solely on yucca seeds, but consume only a small proportion of the developing seeds⁸⁻¹¹. Reciprocal plant specialization has led to mutual dependence between yuccas and their pollinators, as neither species can successfully reproduce without the other^{8,9,12}.

Historically the yucca moth *Tegeticula yuccasella* has been considered a single pollinator species of all but two yucca species¹³, but there is growing recognition that it is actually a large complex of distinct species with narrower host ranges^{9,14,15}. Females with vestigial tentacles that render them unable to handle pollen have been recognized, but have been incorrectly described as a primitively non-pollinating bogus yucca moth (*Prodoxus*)¹⁶ or treated as individual pollinators with failed tentacle development¹³. More recent observations indicate that these moths are a suite of distinct species within the pollinator complex, readily diagnosable on the basis of biological, morphological and molecular traits.

All such cheaters oviposit into fertilized flowers and fruit, and the larvae feed on developing seeds, so they depend on the pollinating moths for subsistence of their larvae. Different species oviposit into the fruits at either of two phenological stages. We will refer to them as 'early' and 'late' cheaters (Fig. 1). Early cheaters co-occur with the pollinators during the latter part of the flowering period but oviposit into the fruit wall a few days after fertilization, exploiting a seed resource unavailable to the coexisting pollinators. In *Yucca filamentosa*, selective abortion based on the number of pollinator eggs causes fruits with >7-8 eggs to abscise within 5 days (ref. 5). Retained fruits in sites without cheaters have average larval densities that cause destruction of 26% of the developing seeds (Fig. 2a); the remainder can not be used by the pollinator because of the selective abortion mechanism. The early cheaters oviposit 3-5 days after fertilization, immediately after the period when the abortion decision is made⁵, so additional eggs do not affect the probability of abortion.

Fruits in populations colonized by early cheaters have average seed destruction levels of 75%, with many fruits being completely devoid of intact seeds (Fig. 2b). Late cheaters use a much-elongated ovipositor to cut into full-size fruit, usually 2-3 weeks after pollination, laying eggs inside seeds or in fleshy mesocarp. The two cheater types are known to coexist in some areas, destroying all seeds in 85% of the fruit in one sample¹⁷.

Phylogenetic analysis was used to study the origins of cheating within yucca moths (Fig. 1). A mitochondrial DNA region of 2.1 kilobases was sequenced for all described *Tegeticula* species, two outgroups, and eight pairs of coexisting pollinator and cheater taxa within the *T. yuccasella* complex. Monophyly was confirmed for the complex, and pollination confirmed as a basal condition within the complex and *Tegeticula* as a whole. Divergence data indicated a period of rapid diversification within the complex, providing modest confidence in exact branching patterns of the very robust terminal clusters. Both of the most parsimonious trees indicated a single origin of the early cheaters and two origins of the late cheaters. The likelihood of this topology, however, is not significantly greater than for those including a single origin of the late cheaters, or even a single origin of all cheaters (Kishino-Hasegawa test¹⁸, $P > 0.05$). Although the cheaters clearly arose from a pollinating ancestor, we will need more data before we can be sure how many times cheating arose.

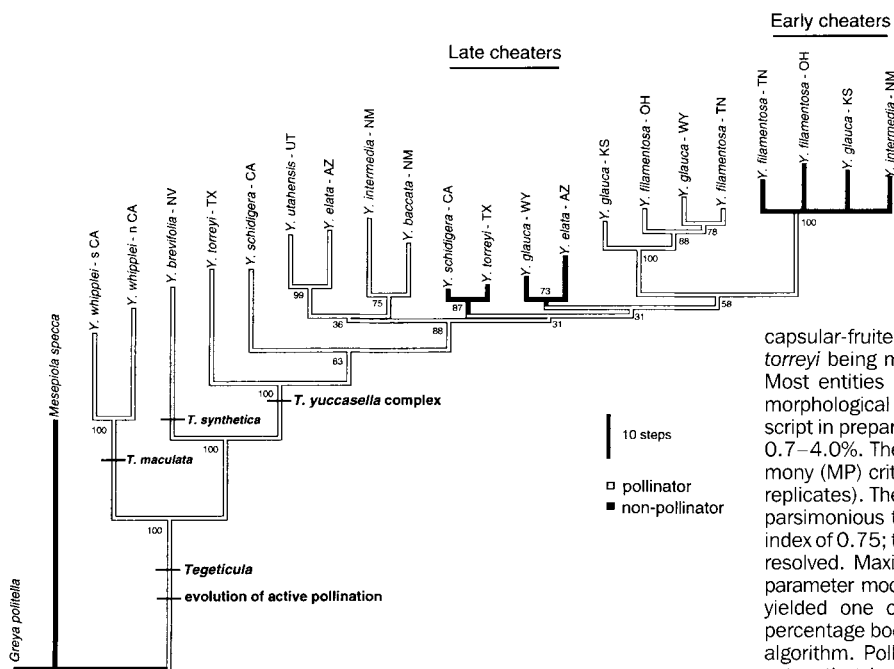
The obligate mutualism appears to have persisted for a long time, despite the very high levels of cost imposed on the plant by the cheaters. Divergence estimates derived using Kimura's two-parameter model¹⁹ suggest that the cheater lineages arose 61-66% of the time since the origin of the *T. yuccasella* complex. The underlying assumption of rate homogeneity was tested and not rejected by comparing maximum-likelihood trees constructed with and without assumption of a molecular clock (Kishino-Hasegawa test¹⁸, $P > 0.05$).

How has the obligate mutualism persisted despite the destabilizing massive increase in cost to the plant imposed by the cheaters? The phylogenetic pattern (Fig. 1) provides important information to resolve this issue. There are two models for the evolution of cheating among pollinators: origin within an exclusive pollinator species on a host, or reversal of mutualism in one pollinator after a host shift has resulted in the coexistence of distinct pollinator species. Simple models of intraspecific reversals of mutualism predict them to be ephemeral, because if there is a cost to pollinating the cheater gene will spread and drive the population to extinction^{20,21}. More complex models incorporating, for example, frequency-dependent selection against cheaters, suggest special conditions that can lead to stable coexistence of pollinators and cheaters^{22,23}. If cheater moths did indeed evolve on their original hosts, coexisting pollinators and cheaters should be sister taxa, but this prediction is not met for any pair in the phylogeny (Table 1 and Fig. 1). Alternatively, whenever two

TABLE 1 Tests of the hypothesis that any of the coexisting pollinator-cheater pairs are sister taxa

Host with constrained pollinator-cheater pair as sister taxa	Ln (likelihood)	Δ Ln (likelihood)
Unconstrained (Fig. 1)	-7215.54	
<i>Yucca elata</i>	-7264.92	49.38 (± 48.90)
<i>Y. filamentosa</i> (OH)	-7379.65	164.11 (± 57.38)
<i>Y. filamentosa</i> (TN)	-7373.87	158.33 (± 50.94)
<i>Y. glauca</i> (KS)	-7364.42	148.88 (± 50.33)
<i>Y. glauca</i> (WY)	-7332.91	117.37 (± 42.42)
<i>Y. intermedia</i>	-7303.12	87.58 (± 55.09)
<i>Y. torreyi</i>	-7266.72	51.17 (± 37.55)
<i>Y. schidigera</i>	-7257.00	41.46 (± 28.31)

Maximum-likelihood values were computed for the unconstrained tree (Fig. 1) and eight ML trees where coexisting pollinator-cheater pairs were constrained as sister taxa in each tree. The right column gives the difference between the constrained and unconstrained trees, with a confidence interval for $P = 0.05$. Because confidence intervals failed to exceed Δ Ln in each case, the null hypothesis that any coexisting pollinator-cheater pair were sister taxa was rejected at the $P < 0.05$ level²⁰.



species of pollinators come to coexist on a host through secondary colonization, either species may come under relaxed selection for pollination. This would be the case, particularly if relative phenology differs, such that one species visits flowers later than the other and so encounters higher frequencies of pollinated flowers. The criterion that two pollinators should coexist on one host is met for several yuccas^{9,13,15}. Furthermore, the present phylogeny already lends support for a history of host shifts within the *T. yuccasella* complex: for example, the late cheaters have diversified onto both fleshy-fruited and capsular-fruited yuccas; the pollinator of the fleshy-fruited *Y. baccata* is nested within a moth clade on capsular-fruited yuccas; and both the early cheaters and their sister group are paraphyletic with regard to hosts. The only documented instance of reversal of mutualism in fig wasps²⁴ corroborates this pattern, in that the cheater coexists with a distantly related pollinator²⁵. Host shifts offer immediate reproductive isolation between the two coexisting taxa, so an emerging cheater gene will only invade one pollinator species, and the mutualism between the other pollinator and the plant remains unaffected.

Historically, obligate mutualists have been thought to shift hosts only rarely, but the phylogenetic data for the yucca moths contradict this suggestion. Host or partner shifts of the type

Early cheaters

FIG. 1 Phylogenetic relationships among pollinating and cheating yucca moths as estimated from a 2.1-kb mitochondrial DNA sequence. *Greya* and *Mesepiola* were chosen as outgroups based on morphological and molecular data²⁷. All *Tegeticula* species are labelled by *Yucca* host and US state. Members of the *T. yuccasella* complex were selected as pairs of coexisting pollinators and cheaters for each taxon; for *Yucca glauca*, two pollinators were included because they coexisted with different cheater taxa. For *Y. baccata* and *Y. utahensis*, known 'late cheaters' were not available for DNA analysis. Hosts of the *T. yuccasella* complex are either

capsular-fruited or fleshy-fruited, with *Y. baccata*, *Y. schidigera* and *Y. torreyi* being members of the monophyletic fleshy-fruited species²⁸. Most entities on different hosts are readily diagnosable based on morphological and biological differences (O.P. and J.L.-M., manuscript in preparation). Sequence divergence²⁰ within the complex was 0.7–4.0%. The data were analysed by applying the maximum-parsimony (MP) criterion²⁹ in a heuristic search with random addition (25 replicates). The topology shown is a strict consensus of the two most parsimonious trees yielded, 761 steps in length, with a consistency index of 0.75; the MP trees differed only in how the sole polytomy was resolved. Maximum-likelihood (ML) analysis assuming a Kimura 2-parameter model of evolution with a transition–transversion ratio of 2 yielded one of the two MP trees. Numbers at the nodes give percentage bootstrap support from 500 replicates using the heuristic algorithm. Pollinator function was mapped as an irreversible character, that is, that pollination would re-evolve once lost was considered impossible.

METHODS. A 2.1-kb mtDNA fragment corresponding to positions 1495–3603 in the *Drosophila yakuba* mtDNA genome³⁰ (across the COI–COII and the intervening tRNA_{Leu}) was amplified by the polymerase chain reaction (PCR) and both strands were directly sequenced. No insertions or deletions were observed, therefore alignment was readily done by eye, yielding 2,103 sites, of which 519 were variable, and 232 were phylogenetically informative. PCR primer sequences and complete sample data are available from the authors. Moth vouchers will be placed in the Smithsonian Institution (USNM). All DNA sequences have been placed in GenBank under accession numbers U49021–U49043.

described here can provide a general mechanism for evolutionary stable escape from many types of obligate mutualism. $\square$

Received 30 August 1995; accepted 16 January 1996.

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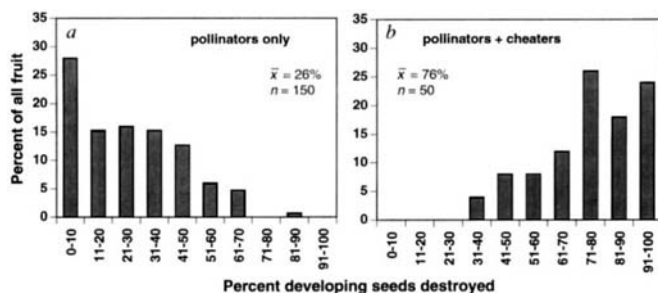


FIG. 2 Levels of seed destruction in populations of *Yucca filamentosa*, a, without and b, with cheaters. Fruits (25) were collected at random after larvae had exited from each of 8 populations in Ohio and Tennessee, and the proportion of developing seeds destroyed by yucca moth larvae was determined by seed counts.

ACKNOWLEDGEMENTS. We thank R. Leuschner and R. Wielgus for moth samples; the National Park Service and State of Texas for permission to collect samples in the Big Bend/Black Gap region; and E. A. Herre, A. Wolfe, and N. Young for valuable comments. This work was supported by grants from the National Geographic Society, NSF, and Vanderbilt University.

Rescue of hybrid sterility in crosses between *D. melanogaster* and *D. simulans*

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THE genetic analysis of reproductive isolation between species of *Drosophila* has now reached the resolution necessary^{1,2} to start answering one of the fundamental questions of evolution: what is the genetic basis of species differences? (ref. 3). A. H. Sturtevant, one of the founders of *Drosophila* genetics, was fascinated by this question⁴ and thought he had found a way to analyse it when he realized that '*Drosophila melanogaster*' was actually two species: *D. melanogaster* and *D. simulans*⁵. By passing genes between these two species he hoped to investigate their genetic differences directly. No doubt he was disappointed to find that the *D. melanogaster*/*D. simulans* hybridization resulted only in unisexual sterile hybrids⁶, a disappointment appreciated all the more by modern evolutionary biologists. Seventy-five years after Sturtevant's description of *D. melanogaster*/*D. simulans* hybrid sterility, we have discovered a strain of *D. simulans* that produces fertile female hybrids in crosses with *D. melanogaster*. Our discovery promises to bring the enormous resolution of *D. melanogaster* genetics to the study of reproductive isolation and species differences.

D. melanogaster and *D. simulans* are both cosmopolitan human commensals⁷. The two species are closely related, differing only by one large and a few small chromosome inversions⁸ and only by about 4–8% on average in DNA sequence^{9–11}. The shape of the male genital arch is the only reliable morphological character that distinguishes the two species⁶. Hybridization between *D. melanogaster* and *D. simulans* has been used primarily to investigate the genetic basis of hybrid inviability^{12–16}. The inviability observed is sex-specific and depends on the direction of the cross: male hybrids die as larvae/pupae from crosses with *D. melanogaster* mothers; female hybrids die as embryos from crosses with *D. simulans* mothers. The embryonic lethality is not fully penetrant and hybrid female escapees can be obtained¹⁶. Strains of *D. melanogaster* and *D. simulans* have been described that rescue either the embryonic (*D. melanogaster*: *In(1)AB*¹⁵, *zhr*¹³; *D. simulans*: *mhr*¹²) or the larval/pupal lethality (*D. melanogaster*: *Hmr*^{15,17}, *In(1)AB*¹⁵; *D. simulans*: *Lhr*¹⁸). There is also strong pre-mating isolation in crosses between *D. simulans* females and *D. melanogaster* males^{6,19}.

While investigating female inviability rescue by the *D. melanogaster* strain *In(1)AB*, we noticed that hybrid females from crosses between *D. simulans* C167.4 females and *In(1)AB* males produced eggs. We confirmed that C167.4 is a *D. simulans* strain by five criteria: (1) normal viability and fertility in crosses with three other strains of *D. simulans*; (2) inviability of hybrid males in crosses

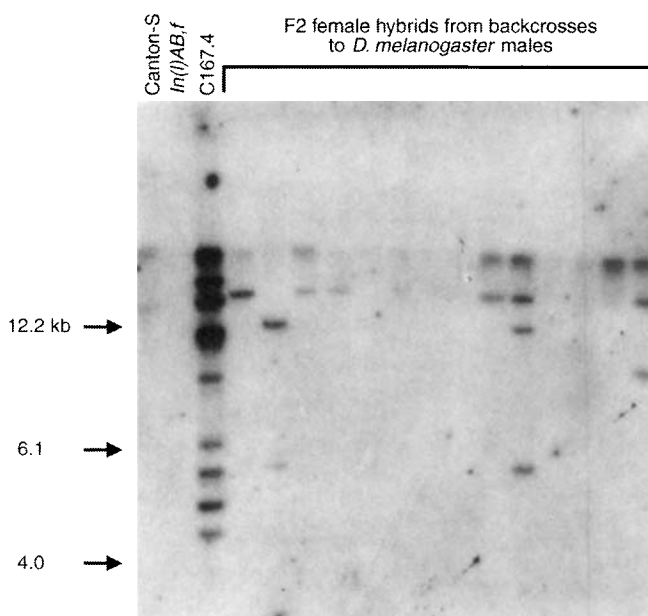


FIG. 2 Southern blot of *D. simulans* C167.4, *D. melanogaster* *In(1)AB*, *f*, *D. melanogaster* Canton-S, and *F₂* backcross female hybrids probed with an internal PCR fragment (659 bp) of the transposable element *mariner*. *F₂* females were collected from the cross of *F₁* C167.4/*In(1)AB*, *f* females X Canton-S *D. melanogaster* males (Table 2). Although *mariner* can transpose in a *D. melanogaster* genetic background²⁴, bands segregating in these *F₂* hybrids were clearly homologous to those present in the *D. simulans* C167.4 strain. Loading: Canton-S and *In(1)AB*, *f*, ~5 ng male genomic DNA; C167.4, ~5 ng female genomic DNA; *F₂* female hybrids, ~0.25 ng from a single female. DNA isolation, radioactive probe synthesis (random hexamer method), and Southern hybridization were done using modifications of standard methods²⁵. DNA was digested with *EcoRI* which cuts outside the *mariner* element. Primers used for *mariner* amplification were: 5'-CGACGACAAAGAGCAGCAG-GAAA-3' and 5'-TCCAGACCACTCATTCGCGC-3' (annealing temperature, 60 °C). PCR was carried out for 30 cycles.

with *D. melanogaster* females; (3) polytene chromosome banding pattern; (4) shape of the male genital arch (Fig. 1); and (5) presence of *mariner*, a transposable element found in *D. simulans* but not *D. melanogaster*²⁰ (Fig. 2).

F₁ hybrids from crosses between *D. melanogaster* and *D. simulans* are sterile and generally have atrophied ovaries or testes^{6,21}. We compared the ovarian and testicular development of *F₁* hybrids produced in interspecific crosses between strains of *D. simulans* (including C167.4) and *D. melanogaster* (Table 1). Crosses between C167.4 females and *In(1)AB* or *In(1)AB*, *f* males (a recombinant derivative of *In(1)AB*) produced female

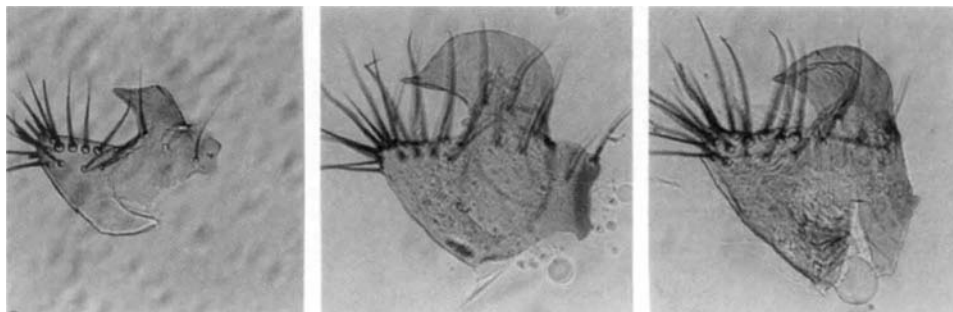


FIG. 1 Male genital arches of left, *D. melanogaster* *In(1)AB*, *f*; centre, *D. simulans* C167.4, and right, an *F₁* hybrid from the cross C167.4 females X *In(1)AB*, *f* males (original magnification, 200×). Genital arches were dissected from males and squashed in lactic acid. Genital arch images were digitized and posterior process areas measured using NIH Image v1.54 image analysis software. Mean posterior process areas ($\mu\text{m}^2 \pm \text{s.e.m.}$) for the three types of males were: 3.717 ± 394 ($n = 24$); 11.736 ± 726 ($n = 24$); and 7.873 ± 595 ($n = 26$) for panels left to right, respectively. The differences in genital arch areas remained after correction for body size (area/wing length). These results are similar to an earlier study by Coyne²³ and confirm that C167.4 is a strain of *D. simulans*.

TABLE 1 Ovarian development in strains of *D. melanogaster*, *D. simulans* and their F_1 hybrids

	Total examined	Proportion with eggs	No. eggs/female (mean $\pm$ s.e.m.)
<i>D. melanogaster</i> :			
<i>ln(1)AB</i>	50	1.00	32.6 $\pm$ 14.0
<i>ln(1)AB, f</i>	30	0.97	20.4 $\pm$ 10.6
<i>D. simulans</i> :			
C167.4	38	1.00	38.7 $\pm$ 17.6
Tsimbazaza	40	1.00	37.8 $\pm$ 9.2
F_1 hybrids (female $\times$ male):			
C167.4 $\times$ <i>ln(1)AB</i>	50	0.94	28.3 $\pm$ 17.1
Oxnard $\times$ <i>ln(1)AB</i>	18	0.55	3.3 $\pm$ 4.3
vermillion $\times$ <i>ln(1)AB</i>	36	0.53	1.4 $\pm$ 1.8
Tsimbazaza $\times$ <i>ln(1)AB</i>	34	0.18	0.6 $\pm$ 2.0
C167.4 $\times$ <i>ln(1)AB, f</i>	50	0.88	11.3 $\pm$ 13.5
Oxnard $\times$ <i>ln(1)AB, f</i>	46	0.56	4.5 $\pm$ 12.5
vermillion $\times$ <i>ln(1)AB, f</i>	42	0.57	3.0 $\pm$ 5.1
Tsimbazaza $\times$ <i>ln(1)AB, f</i>	50	0.10	0.5 $\pm$ 2.1
C167.4 $\times$ <i>zhr</i>	50	0.28	1.4 $\pm$ 3.3
C167.4 $\times$ Oregon-R*	43	0.81	10.0 $\pm$ 9.5
C167.4 $\times$ Melbourne†	15	0	0
<i>ln(1)AB</i> $\times$ C167.4	31	0.42	1.0 $\pm$ 2.2
<i>ln(1)AB</i> $\times$ Oxnard	50	0.12	0.14 $\pm$ 0.4
<i>ln(1)AB</i> $\times$ vermillion	50	0.14	0.2 $\pm$ 0.6
<i>ln(1)AB</i> $\times$ Tsimbazaza	41	0.00	0
<i>ln(1)AB, f</i> $\times$ C167.4	50	0.02	0.04 $\pm$ 0.28
<i>ln(1)AB, f</i> $\times$ Oxnard	50	0.00	0
<i>ln(1)AB, f</i> $\times$ vermillion	50	0.00	0
<i>ln(1)AB, f</i> $\times$ Tsimbazaza	50	0.00	0
Controls (<i>mel</i> $\times$ <i>sim</i>)‡	641	0.00	0
Test for cytoplasmic effect			
[C167.4]§ $\times$ <i>ln(1)AB, f</i>	154	0.54	3.7 $\pm$ 7.4
[Tsimbazaza] $\times$ <i>ln(1)AB, f</i>	129	0.53	3.6 $\pm$ 6.8

D. simulans strains used: C167.4 (Nanyuki, Kenya), Tsimbazaza (Madagascar), vermillion, and Oxnard (California). *D. melanogaster* strains used: *ln(1)AB* (= *ln(1)9E*; 13E), *ln(1)AB, f* (a recombinant derivative of *ln(1)AB* carrying the bristle mutation *forked*), *w m f*, Melbourne (Australia), and Antigua (Caribbean). Crosses between *D. simulans* females and *D. melanogaster* males were carried out in crowded conditions (up to 100 pairs per 4-inch vial) to encourage mating. For other crosses 10 pairs were sufficient. Flies were cultured on standard *Drosophila* cornmeal media at 25 °C. Ovaries were dissected from 5–7-day-old virgin females in *Drosophila* Ringer's solution and examined using differential interference microscopy.

* This cross was carried out at 18 °C to obtain hybrid female escapees, which normally die as embryos at higher temperatures¹⁶. Virgin females were dissected after 7–10 days to allow for temperature effects on the rate of ovarian development²².

† The female inviability rescue activity of the *D. melanogaster* Melbourne strain was discovered during these experiments. The rescue activity has not been localized.

‡ Summary of hybridizations between females from 3 *D. melanogaster* strains (Melbourne, Antigua, and *w m f*) and males from 4 *D. simulans* strains (C167.4, Tsimbazaza, Oxnard, vermillion).

§ Brackets denotes origin of *D. simulans* cytoplasm. We performed reciprocal crosses between C167.4 and a second *D. simulans* strain, Tsimbazaza. Female progeny from both crosses are heterozygous for C167.4 and Tsimbazaza but differ in the origin of their cytoplasm. These females were hybridized to *D. melanogaster ln(1)AB, f* males. No significant difference in egg production was observed for F_1 females that had cytoplasm of C167.4 or Tsimbazaza origin ($P = 0.91$, two-tailed t-test).

hybrids with well developed ovaries which contained large numbers of mature eggs. Lower levels of hybrid female fertility were also observed in crosses involving two additional strains of *D. simulans*, Oxnard and vermillion, to *ln(1)AB* or *ln(1)AB, f* males. These results indicate a strain-dependent continuum of hybrid female fertility in crosses between *D. simulans* females and *D. melanogaster* males. No hybrid male sterility rescue was observed. All F_1 hybrid males from all crosses had rudimentary

TABLE 2 F_2 progeny from backcrosses of F_1 C167.4, $f^+/ln(1)AB, f$ hybrid females to *D. melanogaster* or *D. simulans* males

Backcross male	No. F_2 females		No. F_2 males	
<i>D. melanogaster</i>	<i>f</i>	<i>f⁻</i>	<i>f</i>	<i>f⁺</i>
<i>ln(1)AB, f</i>	25	20	11	11
Oregon-R*		65	45	12
Canton-S*		19	16	2
<i>D. simulans</i>				
C167.4*	200		0	105

* Genotype of females with respect to the *f* marker unknown in backcrosses to Oregon-R, Canton-S, and C167.4.

testes with no advanced stages of spermatogenesis (1,131 F_1 males dissected from crosses listed in Table 1).

The fact that hybrid females from the cross of *D. simulans* females to *D. melanogaster* males are rare undoubtedly explains why their fertility has gone unnoticed until now. It also raises the question of whether the female sterility rescue is caused by an interaction between the C167.4 sterility rescue gene(s) and the female inviability rescue mutation(s) carried by the *ln(1)AB* strain. Three crosses in Table 1 address this issue. First, hybrid females were sterile in crosses between C167.4 females and *zhr* males, another *D. melanogaster* mutation which rescues hybrid female inviability¹³ (Table 1). Second, hybrid females were fertile in crosses between C167.4 females and *D. melanogaster* Oregon-R males (Table 1). Oregon-R does not carry an inviability rescue mutation¹³. These F_1 females were lethal escapees obtained by crosses carried out at 18 °C. Third, we discovered that the *D. melanogaster* wild-type strain Melbourne carries an unmapped female inviability rescue mutation. Hybrid females from crosses between C167.4 females and Melbourne males were sterile (Table 1). These three crosses indicate that the C167.4 sterility rescue is not dependent on inviability rescue *per se*.

The reciprocal cross of *ln(1)AB* or *ln(1)AB, f* females to C167.4 males did not produce fertile hybrid females indicating a maternal effect for the female sterility rescue (Table 1). To determine if this maternal effect was of cytoplasmic or nuclear origin, we replaced the cytoplasm of C167.4 with cytoplasm from Tsimbazaza, a *D. simulans* strain that gave no female sterility rescue (Table 1), and crossed these females to *ln(1)AB, f* males. No significant difference in ovarian development was observed between hybrid females which differed only in the source of *D. simulans* cytoplasm (Table 1). Therefore, the hybrid female sterility rescue is due to maternal contributions from the nuclear genome of C167.4. The ovarian egg counts in this set of crosses, however, were $\sim 1/3$ of that observed for F_1 females from the C167.4 female $\times$ *ln(1)AB, f* male cross in Table 1. This observation indicates that the mutation(s) responsible for the sterility rescue of the C167.4 strain is not dominant.

The discovery of hybrid fertility between *D. simulans* and *D. melanogaster* raises the hope that the resolution of *D. melanogaster* genetics can be brought to the study of reproductive isolation and species differences. To address these questions fully, we need to be able to introduce genes from *D. simulans* into *D. melanogaster* and vice versa. Our data suggest that it may be possible to transfer genes between these two species by repeated generations of backcrossing (that is, introgression). F_1 females from crosses between C167.4 females and *ln(1)AB, f* males produced F_2 progeny in backcrosses to either *D. simulans* or *D. melanogaster* males (Table 2). In backcrosses to *D. melanogaster* males, F_2 progeny were segregating for the *D. simulans f⁺* marker, suggesting that this region of the *D. simulans* X-chromosome might be introgressable into *D. melanogaster*. We used the transposable element *mariner* to probe Southern blots of F_2 hybrids from backcrosses to *D. melanogaster* (Fig. 2). Because *mariner* is absent from *D. melanogaster* populations²⁰, it is a useful dominant marker for regions of the *D. simulans* genome segregating in a *D.*

melanogaster background. *mariner* bands are clearly segregating in our F₂ backcrosses to *D. melanogaster* (Fig. 2). Polytene chromosome squashes of F₂ female hybrids from backcrosses to C167.4 were also analysed. Of 14 F₂ females examined, one was heterozygous for the *D. melanogaster* X-chromosome inversion *In(1)AB*, suggesting that introgressions from *D. melanogaster* into *D. simulans* may also be possible. All strains discussed here are available from J.R. □

Received 2 November 1995; accepted 12 January 1996.

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ACKNOWLEDGEMENTS. We thank M. Akam and D. Stern for use of their digitizer and S. Collier, S. Russell, D. Gubb and G. Hurst for discussion. Supported by grants from the BBSRC to M.A., from the British Council to K.S. and from the European Union to S.H.

Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice

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SPERMIOGENESIS is a complex process by which postmeiotic male germ cells differentiate into mature spermatozoa. This process involves remarkable structural and biochemical changes including nuclear DNA compaction and acrosome formation^{1,2}. Transcriptional activator CREM (cyclic AMP-responsive element modulator) is highly expressed in postmeiotic cells^{3–5}, and CREM may be responsible for the activation of several haploid germ cell-specific genes involved in the structuring of the spermatozoon^{6–7}. The specific role of CREM in spermiogenesis was addressed using CREM-mutant mice generated by homologous recombination. Analysis of the seminiferous epithelium in mutant male mice reveals postmeiotic arrest at the first step of spermiogenesis. Late spermatids are completely absent, and there is a significant increase in apoptotic germ cells. We show that CREM deficiency results in the lack of postmeiotic cell-specific gene expression. The complete lack of spermatozoa in the

mutant mice is reminiscent of cases of human infertility.

We have previously documented the differential regulation of the CREM gene during spermatogenesis^{3–5}. A striking developmental switch³ in CREM expression leads to the accumulation of large amounts of the protein from the round spermatid stage onwards⁵. Various candidate target genes for CREM transcriptional activation in postmeiotic germ cells have been identified, including protamine⁵, caldesmon⁶ and transition protein-1 (refs 5, 7), which are required for the structuring of the spermatozoon².

To address the developmental and physiological role of CREM we generated mutant mice with a gene disrupted by homologous recombination. We constructed a targeting vector in which a portion of the CREM 3'-terminal exon encoding the DNA binding domains was replaced by a pGk-neomycin cassette (Fig. 1a, b). The construct was selected to inactivate all the numerous CREM isoforms^{8,9}. Indeed, the CREM gene generates a large family of transcripts by alternative splicing, alternative promoter usage, and alternative translational initiation^{10,11}. However, all the CREM isoforms share the same DNA binding-dimerization domains¹¹. Analysis of RNA from testis (Fig. 1c) and other organs (not shown) from heterozygous and homozygous CREM-deficient mice showed reduced and absent expression of CREM transcripts, respectively. Western blot analysis using CREM antibodies⁵ confirmed the RNA data (Fig. 1d). That there were no CREM products in the mutant mice is consistent with the previously described strict regulation of transcript stability by post-transcriptional modifications⁴.

Reduced fertility of the heterozygous mice was observed. The average litter size in the heterozygote/heterozygote cross (4.9 ± 0.3 ; $n = 36$) was significantly different ($P < 0.01$) from the litter size for the wild-type male/heterozygote female cross (7.2 ± 0.6 ; $n = 13$), from the litter size of the wild-type female/heterozygote male cross (6.2 ± 0.5 ; $n = 12$), and from that of the wild-type/wild-type cross (7.1 ± 0.3 ; $n = 8$). Comparison of the homozygous CREM-deficient mice with their normal and heterozygous littermates indicated no macroscopic physical aberrations or reduction in body weight. Similarly, there were no apparent differences in the structure of internal organs (not shown). However, the weight of the testes in the CREM-deficient mice was reduced by 20–25% (Fig. 24). Comparison of the seminal fluid of heterozygous mice with normal littermates demonstrated a 46% reduction in the number of spermatozoa, a 35% decrease in the ratio of motile spermatozoa, and a twofold increase in spermatozoa with aberrant structures. Most aberrant spermatozoa were characterized by a kink and bubble-like structure midway along the tail (Table 1). Strikingly, the seminal fluid from homozygous CREM-deficient mice completely lacked spermatozoa (Table 1). This result demonstrates dramatic impairment of spermatogenesis in the CREM-deficient mice. The homozygous males are indeed sterile; the homozygous females were fertile with normal ovary structure (not shown).

To determine the nature of the sperm deficiency we performed a detailed anatomical analysis of the seminiferous epithelium. Consecutive spermatogenic cycles are classically depicted as waves of differentiating germ cells¹². In the mouse, each wave is divided into 12 stages, representing specific cellular associations^{1,13}. Tubular segments containing postmeiotic germ cells undergoing spermiogenesis appear as dark sections under transillumination because of higher DNA compaction¹². Tubuli from CREM-deficient mice have a diameter reduced by 20–30% and completely lack the normal spermatogenic wave and the corresponding dark sections (Fig. 2B and data not shown). Squash preparations¹⁴ from consecutive segments of the seminiferous epithelium demonstrate that spermatogenesis in the CREM-deficient mice is interrupted at the very early spermatid stage (Fig. 2C). Neither elongating spermatids nor spermatozoa are observed, but somatic Sertoli cells appear normal. In contrast, tubules from heterozygous mice displayed a normal spermatogenic wave and cellular composition (data not shown).

In the CREM-mutant mice, we observed many multinucleated

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giant cells, normally infrequent in the wild type¹⁵ (Fig. 2C); these bodies have previously been shown to correspond to apoptotic cells¹⁵. For confirmation we used *in situ* terminal transferase 3'-end labelling¹⁶ to indicate high levels of DNA free ends, a diagnostic feature of apoptotic cells. The number of apoptotic cells in the CREM-deficient mice is 10-fold higher than in normal mice (Fig. 2E); heterozygous mice displayed a normal frequency of apoptotic cells. This indicates that the lack of CREM causes germ cells to cease differentiation and to undergo apoptosis.

Histological analysis of the testis from CREM-deficient mice confirmed a reduced tubule diameter and the corresponding absence of developing spermatids and spermatozoa (Fig. 2D). The somatic Sertoli and Leydig cells appeared normal, and numerous apoptotic cells were clearly visible in tubules of stages VII–X (Fig. 2D). Furthermore, during prepubertal development, apoptotic cells appear coincidentally with the maturation of haploid spermatids in CREM-deficient mice (not shown).

Given the importance of the activation of specific postmeiotic genes in spermiogenesis, and the involvement of CREM in their regulation⁵, we examined the expression of a battery of genes in the CREM-deficient and normal mice (Fig. 3a). CREM has an important role in the activation of genes such as those for

TABLE 1 Sperm count and motility

	+/+	+/-	-/-
Number of spermatozoa per mouse ($\times 10^7$)	23 $\pm$ 4	12 $\pm$ 1*	0
Aberrant structure (%)	11 $\pm$ 3	21 $\pm$ 2*	
Motility (% moving spermatozoa)	65 $\pm$ 5	43 $\pm$ 6*	

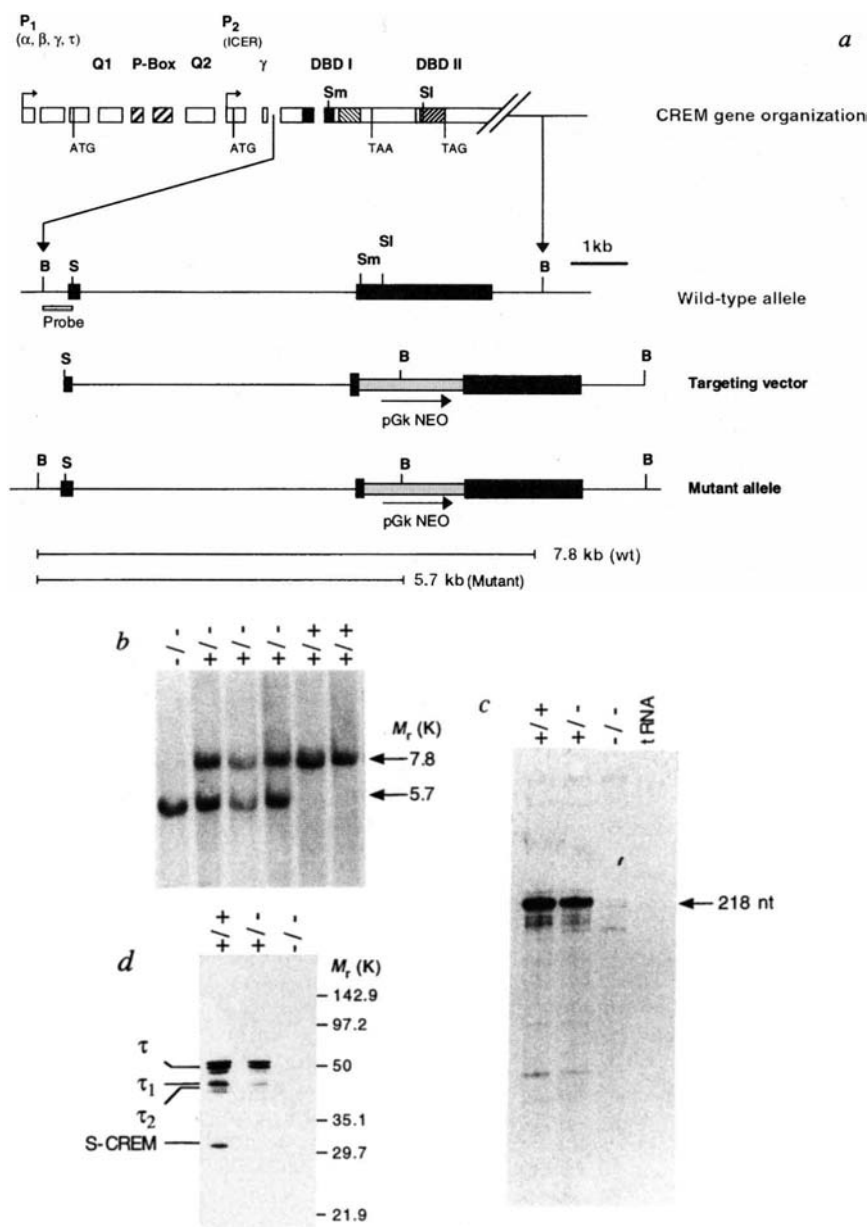
Semen was extracted from the vas deferens and epididymis, and spermatozoa counted with an haemocytometer. The proportion of motile spermatozoa was calculated by observation with a microscope connected to a video camera.

* $P < 0.05$.

protamine 1 and 2 and transition proteins 1 and 2. The lack of expression of these genes may explain the impairment of spermiogenesis in the CREM-deficient mice, or may reflect cell loss caused by an earlier block in differentiation. Moreover, expression of caldesmon⁶, *Krox-20* (ref. 17) and *Krox-24* (ref. 18) is strongly reduced in both the heterozygous and homozygous mutant animals. In contrast, other genes expressed in premeiotic germ cells,

FIG. 1 Targeted disruption of the CREM gene. **a**, Strategy for generating the CREM null mutation. A 12-kb phage genomic clone including the two 3'-terminal CREM exons was isolated. A neomycin-resistance gene under the control of the phosphoglycerate kinase promoter (pGk NEO) was inserted between the *Sma*I (Sm) and *Sa*II (SI) sites straddling the two alternative DNA-binding domains (DBD I and II), thereby inserting an in-frame stop codon and introducing an internal *Bam*HI site (B). For subsequent Southern blot analysis, a 400-bp *Bam*HI–*Sa*II (S) fragment at the 5'-end of the genomic clone was used as a probe external to the targeting vector. **b**, Hybridization of the probe with *Bam*HI-digested genomic DNA yielded 7.8-kb (wild type) and 5.7-kb (mutant) bands. Germline transmission of the mutation followed normal mendelian inheritance. **c**, RNase protection analysis of CREM mRNA expression in testis. Total testis RNA (5 μ g) from wild-type (+/+), heterozygote (+/-) or homozygote mutant (-/-) littermates or tRNA control (10 μ g) was hybridized with the probe 6/N1 (ref. 3). A 218-nucleotide fragment corresponds to specific protection by the testis CREM transcripts. **d**, Western blot of testis total protein extracts using a CREM-specific antibody. Bands corresponding to CREM τ , CREM τ 1, CREM τ 2 and S-CREM^{5,11}, only visible in the wild type and heterozygote testis, are indicated (τ , τ 1, τ 2, S-CREM, respectively).

METHODS. CREM genomic clones were isolated from a C57BL/6, λ EMBL3 partial *Sau*3AI library. A 12-kb *Bam*HI fragment was subcloned into pBlue-script SK⁺ for subsequent insertion of the neomycin resistance cassette. Embryonic stem cells (H1 line) were electroporated with the targeting construct and four positive clones were established. Four clones were injected into C57BL/6 blastocysts. Male chimaeras were bred with C57BL/6 mice and germline transmission was obtained with one clone. All mice were bred into a 129/Sv genetic background. Genomic DNA was isolated from tail biopsies from two-week-old animals.



such as that for proacrosin binding protein¹⁹ and *Hoxa-1* (ref. 20), were expressed normally in the mutant mice.

Importantly, CREB expression is unchanged with respect to normal littermates (Fig. 3b). CREB is the only other cAMP-responsive factor to be detected in male germ cells²¹. However, these CREB isoforms are C-terminally truncated, lacking DNA

binding–dimerization function, and their physiological role remains unclear²¹. Furthermore, the expression of ATF-1, the only other cAMP-responsive family member directly phosphorylated by PKA^{11,22}, appears unchanged in various tissues (Fig. 3d and data not shown). Levels of the co-activator CREB binding protein (CBP)²³ (Fig. 3c) and activating transcription factor-2 (not

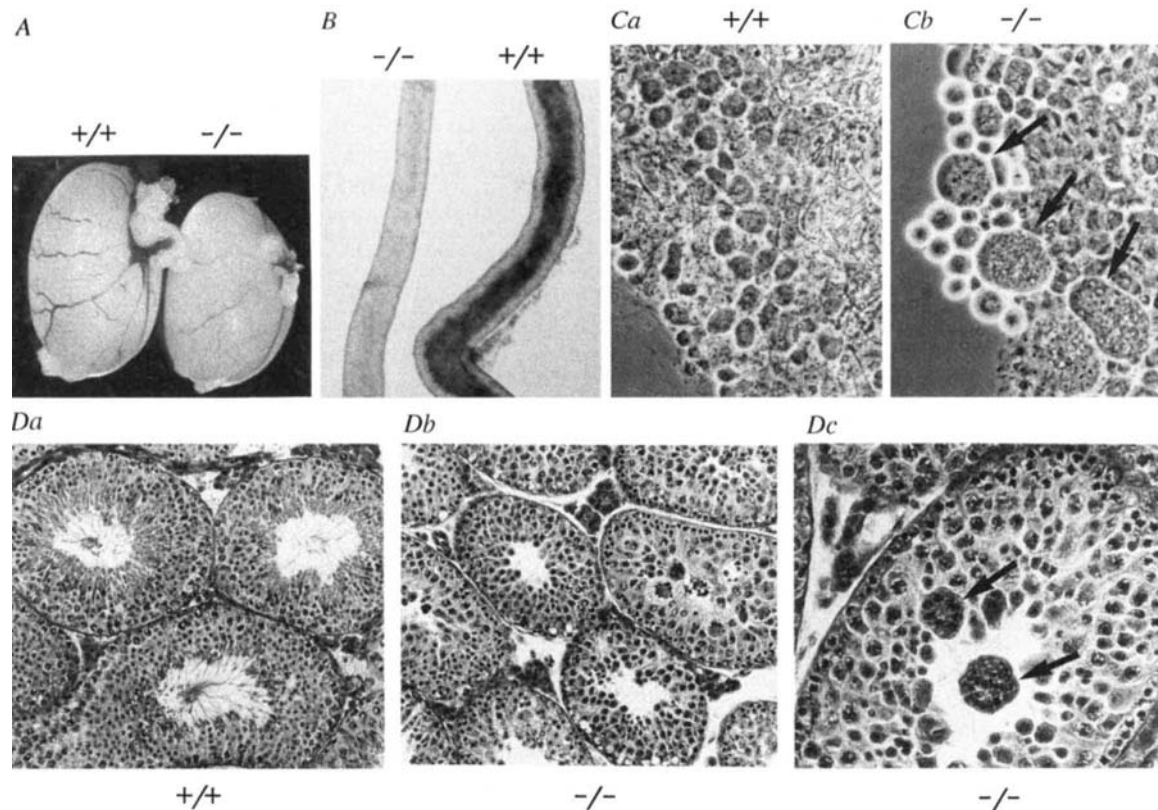
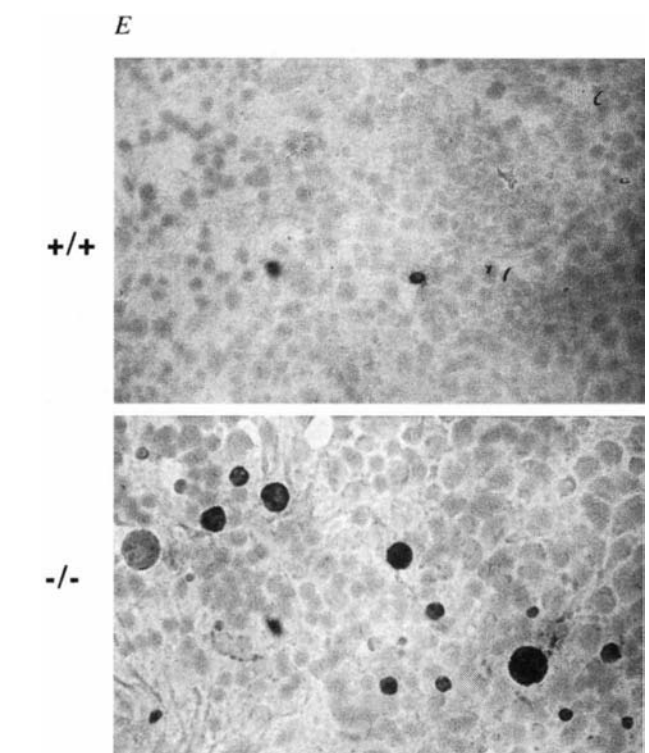


FIG. 2 CREM deficiency causes spermiogenesis arrest and male germ-cell apoptosis. **A**, Testes from an eight-week-old homozygous mutant ($-/-$) and a wild-type ($+/+$) littermate. Note a 20–25% reduction in size of the mutant testis and reduced vascularization compared to wild type. **B**, Transilluminated seminiferous tubules dissected from the testis of eight-week-old homozygous mutant ($-/-$) and wild-type ($+/+$) littermates. The dark section visible in the wild-type tubule corresponds to regions of spermiation (stages VII–VIII)¹², and is completely absent from the homozygous mutant tubules, which are also reduced in diameter. (Magnification, $\times 10$). **C**, Squash preparations¹⁴ of seminiferous tubule segments from wild-type ($+/+$) and mutant ($-/-$) testis viewed under phase-contrast illumination. (Magnification, $\times 400$). Arrows indicate multinucleated giant cells in the $-/-$ mice not visible in tubules from either wild-type (**a**) or heterozygous mice. Developing spermatids and spermatozoa are completely absent from the epithelium of mutant mice. **D**, Histological analysis of testis sections from eight-week-old wild-type (**a**) and mutant (**b**) littermates. (Magnification, $\times 200$). **c**, Mutant testis. (Magnification, $\times 400$). Arrows indicate the characteristic multinucleated giant cells. **E**, *In situ* detection of apoptotic cells. Squash preparations of seminiferous tubules were labelled with terminal deoxynucleotidyl transferase and digoxigenin-ddUTP. (Magnification, $\times 400$). Sections are analogous to those in **C**; dark-staining bodies represent apoptotic cells, the nuclei of which contain high concentrations of DNA free ends, and correspond to the multinucleated giant cells indicated in **C** and **D** as well as smaller, highly refractile bodies visible in **C**. Apoptotic cells were counted in tubule sections from two testes of two different $+/+$, $+/-$ and $-/-$ mice. Results of this count are: for $+/+$ mice, 9.6 ± 1.2 apoptotic cells per mm; for $+/-$ mice, 9.3 ± 0.9 cells per mm; and for $-/-$ mice, 103 ± 5.2 cells per mm. The heterozygous mice displayed the same testis phenotype as the wild type.

METHODS. Seminiferous tubules were dissected and photographed under a transillumination dissection microscope. Microdissection of tubules into 1-mm segments was performed using the improved transillumination method¹⁴. Cover-slip-squashed sections were prefixed in liquid nitrogen and then fixed in 10% formalin. *In situ* detection of apoptotic cells was performed as previously described¹⁵. For histological analyses, freshly



dissected testes were immersed in Bouin solution. Sections were prepared according to standard procedures and stained using haematoxylin/eosin.

shown) also appear unchanged. This demonstrates that no redundant function can substitute for CREM in germ cells.

We have shown that CREM is essential for the differentiation program of spermiogenesis. The characteristic expression of CREM in neuroendocrine cells begs the question as to additional

physiological consequences of its ablation. Detailed analyses are in progress to resolve this point. It should be stressed, however, that the increased apoptosis of germ cells in the CREM-deficient mice (Fig. 2E) is consistent with recent results linking CREM to cell-cycle gene regulation²⁴ and the induction of apoptosis in certain cell types (unpublished observations). Spermatogenic arrest, as exhibited in CREM-deficient mice, is also a feature of many cases of infertility in human males^{25,26}. Thus these mice may constitute a tool for determining the altered physiological processes in human male infertility. □

Received 26 October; accepted 29 December 1995.

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ACKNOWLEDGEMENTS. We thank E. Borrelli, A. M. Jelen, E. Lalli, B. Jégou, T. Lufkin, F. Rijli, E. Zazopoulos, M. Lamas, L. Penna, K. Tamai, S. Vyas, M. Lanotte, S. Ruchaud, J.-M. Garnier and A. Saiardi for advice, discussions and material; and E. Heitz, R. Matyas, B. Boulay and P. Goetz-Renier for technical help. F.N. is supported by a fellowship from the Medical Research Council of Canada; L.M. is supported by a short-term EMBO fellowship. This work was supported by grants from CNRS, INSERM, CHUR, FRM, the Association pour la Recherche sur le Cancer, and Rhône-Poulenc Rorer.

Severe impairment of spermatogenesis in mice lacking the CREM gene

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SPERMATOGENESIS is a complex developmental process that occurs in several phases. A large number of genes have been identified that are expressed during spermatogenesis^{1,2}, but the biological significance of many of these is not yet known. We have used gene targeting to selectively eliminate the transcription factor CREM (cyclic AMP-responsive element modulator), which is thought to

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FIG. 3 Analysis of gene expression in CREM-deficient mice. *a*, Analysis of mRNA expression of protamines 1 and 2 (refs 27,28) transition proteins 1 and 2 (TP1, TP2) (refs 29,30), caldesmon (ref. 6), proacrosin binding protein (ref. 19), *Krox-20* (ref. 17), *Krox-24* (ref. 18), *Hoxa-1* (ref. 20) and *G6PD* (ref. 3) by reverse transcription–polymerase chain reaction (RT–PCR) analysis. Total testis RNA (1 µg) from wild-type (+/+), heterozygote (+/–) and homozygous mutant (–/–) mice was used in each analysis. *b*, *c*, Western blot analysis of CREB and CBP expression in a range of tissues from wild-type (+/+), heterozygote (+/–) and homozygous mutant (–/–) littermates. No changes in expression of CREB or CBP proteins is detectable. *d*, Analysis of ATF-1 mRNA expression by quantitative RT–PCR in a range of tissues from normal and mutant mice. Expression was not detected in testis samples and was unchanged in the other tissues examined. Ad, adrenal; Cb, cerebellum; Lv, liver; Sp, spleen; Ts, testis. METHODS. Quantitative RT–PCR was performed using standard methods. The quality of RNA was checked for each reaction. Equivalent results were obtained from five different sets of mutant and normal mice. Primers were selected within the coding sequence of each gene according to standard criteria (their sequence is available from the authors on request). The identity of amplified fragments was confirmed by Southern blot analysis using specific internal ³²P-labelled oligonucleotide probes. Analogous results were obtained by northern analysis. Polyclonal anti-CREB antiserum was obtained from Upstate Biotechnology (New York). Polyclonal anti-CBP antiserum was raised against an N-terminal peptide derived from the published CBP sequence²³.

be important for mammalian spermatogenesis³⁻⁵. Male mice deficient for all CREM proteins are sterile, as their developing spermatids fail to differentiate into sperm, and postmeiotic gene expression in the testis declines dramatically. The cessation of sperm development is not accompanied by decreases in the levels of follicle-stimulating hormone or testosterone. Our findings indicate that the CREM gene is essential for spermatogenesis, and mice deficient for this transcription factor could serve as a model system for the study of idiopathic infertility in men.

The vector used to target the CREM gene in embryonic stem (ES) cells is shown in Fig. 1a. This deletion disrupts the gene and removes the coding information for both of the leucine zipper domains. Leucine zipper domains have been shown to be essential for dimerization and subsequent DNA binding (for review see ref. 6). Four independent ES cell clones were used to generate chimaeric mice. Two clones gave rise to germline-transmitting mice, which were crossed with C57 BL/6 mice, and the resulting heterozygotes were interbred. By using Southern blot analysis we have genotyped 97 F₂ offspring and found a distribution of 24 +/+ to 41 +/- to 32 -/-, which does not deviate significantly from the expected mendelian ratio. The complete inactivation of the CREM gene was confirmed by Southern blot analysis (data not shown) and RNase protection analysis (Fig. 1b). The expression of the CREM gene was visualized by β -galactosidase staining (Fig. 1c), demonstrating activation of the gene primarily in post-meiotic germ cells. Mice deficient for the CREM gene appear healthy and exhibit no obvious impairment of growth or development, although homozygous mutant males are unable to reproduce despite normal mating behaviour. In contrast, homozygous mutant females are fertile.

Preliminary analysis of testis from CREM-mutant mice indicated a dramatic reduction in size and weight compared with littermate controls (Table 1). Reduced testicular size may reflect a disturbance of the spermatogenic process that, in turn, is primarily controlled by pituitary gonadotropic hormones and testicular androgens⁷. Further analysis demonstrated that circulating and testicular androgens, as well as follicle-stimulating hormone (FSH), are not reduced in CREM-mutant males (Table 1), eliminating hormonal deficiency as a cause for infertility. In addition, the presence of normal male genitalia and successful

mating behaviour indicate that these animals have normal hormone levels. However, histological examination revealed a complete and highly specific interruption of spermatogenesis. At all time points of evaluation, early haploid germ cells (round spermatids) were the most advanced germ cells present, but they failed to undergo morphological differentiation and development into elongated spermatids (testicular sperm) (Fig. 2b-d). The complete absence of testicular sperm and of spermatozoa in epididymal sections (Fig. 2f) explains why CREM-mutant mice are infertile despite normal sexual behaviour and copulation. Most of the round spermatids present in the testis of homozygous mutant mice exhibit normal morphology, whereas some cells form multinucleated cells, which in some cases are in the process of detaching from the seminiferous epithelium (Fig. 2b, d). These cells may be apoptotic spermatids, as demonstrated in ref. 8. All spermatids resulting from second meiotic division were blocked in further development. In some tubules, spermatid development, staged on the basis of their acrosome development, advanced to step 4, and very occasionally to step 5. In other tubules, however, spermatid development was already arrested in step 1. In addition, round spermatids and multinucleated cells were observed in large numbers in the epididymis (Fig. 2f). This indicates that round spermatid development continues for at least 8-10 spermatogenic

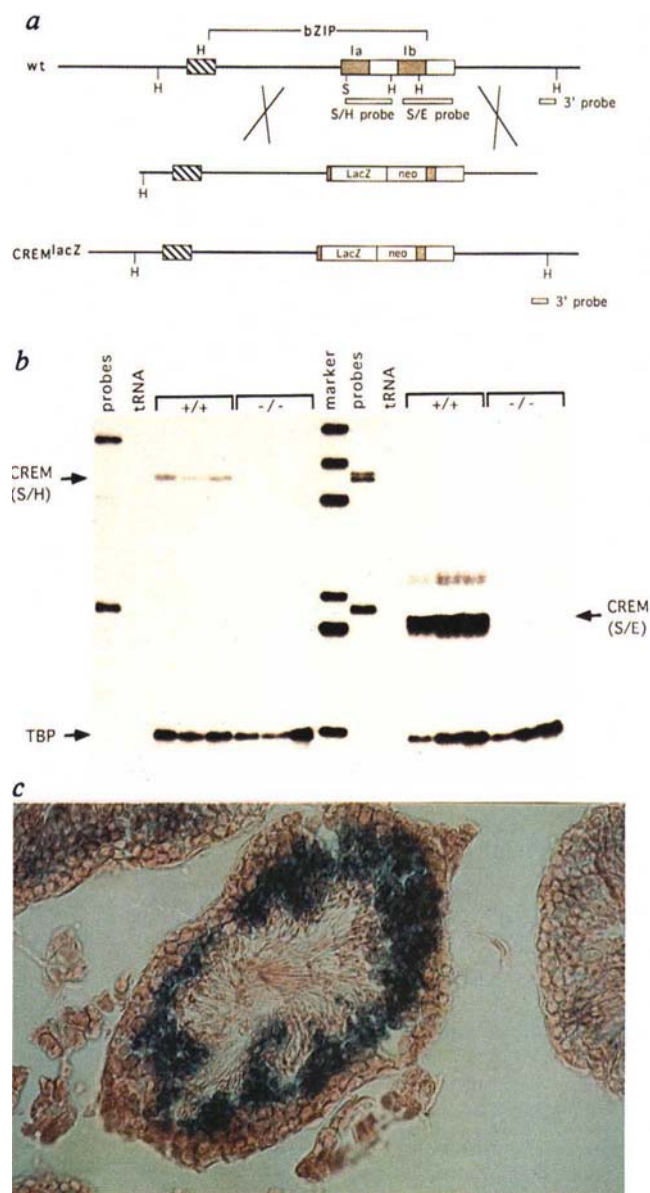


FIG. 1 Inactivation of the murine CREM gene. *a*, Targeting strategy. The top line indicates a portion of the CREM locus with exons H and Ia/Ib, which encode the two alternative DNA binding domains. H and S indicate *Hind*III and *Sma*I sites. The middle line shows the targeting construct. Neo and LacZ indicate neomycin-resistance and β -galactosidase genes, respectively. The bottom line (CREM^{lacZ}) shows the targeted CREM locus in which the DNA binding domains have been replaced by the LacZ-neo fusion cassette. *b*, RNase protection analysis. Testis RNA samples from wild-type (+/+) or mutant (-/-) mice were analysed for CREM gene expression with the two probes indicated in *a* as well as a probe for TATA-box binding protein (TBP)²⁴. The arrows point to the protected fragments. No CREM RNA containing either of the DNA binding domains is detectable in the mutant animals, and the level of TBP mRNA is unchanged. *c*, β -galactosidase staining of a testis from a +/+ mouse demonstrates CREM expression in round spermatids.

METHODS. CREM DNA was cloned from a strain 129 genomic library. The targeting construct contains 5.5- and 2.2-kb homology fragments inserted into the pHM3 vector²⁵. The construct was linearized with *Not*I, and targeted cells (8 of 90) were produced according to standard methodologies. Homologous recombinant clones were identified by Southern blot analysis of *Hind*III-digested DNA with the 3' probe indicated in *a* and confirmed with several inside and outside probes (not shown). The targeted ES cells were injected into blastocysts to generate chimaeras. Two of the chimaeras mated transmitted the mutant CREM gene to their agouti F₁ offspring. F₁ mice (+/-) having the CREM mutation were bred to obtain +/+ and -/- F₂ mice. Testis RNA from 10-week-old mice was prepared and analysed by RNase protection assay according to standard methods, and β -galactosidase staining was according to standard methods.

cycles, despite the lack of the CREM gene, and that the accumulation of these germ cells is overcome by shedding them into the tubular lumen and subsequently to the epididymis.

To investigate the effect of CREM deficiency on potential downstream genes, we analysed testis RNA from wild-type and mutant mice for several well-studied testis markers. Messenger RNA for proacrosin, an acrosomal serine protease⁹⁻¹¹, is slightly reduced in the mutant mice, which may reflect the reduction in expression of proacrosin in spermatid cells rather than in pachytene spermatocytes, which are present in expected numbers in the homozygous mutant mice.

Protamines are small arginine-rich proteins that enter the nucleus and aid in the compaction of the mammalian sperm head during the later stages of spermatogenesis¹². Transition protein-1 (Tp-1), a chromosomal protein, and protamines are highly basic and have a similar control pattern for expression, which involves transcription in early postmeiotic cells (round spermatids) and translational regulation that postpones synthesis of the encoded proteins for several days^{13,14}. The mitochondrial capsule selenoprotein (MCS) is a major structural protein of the keratinous mitochondrial capsule in mammalian sperm. This capsule functions in shaping mitochondria into the helical sheath surrounding the flagellum¹⁵. Similar to protamine 1 and Tp-1, mRNAs encoding MCS are translationally repressed with long poly(A) tracts in early spermatids and are translationally active in late spermatids^{13,16}. The RNA levels for all of these markers tested (MCS, protamine 1 and Tp-1) are dramatically reduced in homozygous mutant mice (Fig. 3), but are only slightly reduced in heterozygous mice (data not shown). It should be noted that heterozygous mice exhibit similar behaviour to wild-type mice with regard to mating and fertility. The promoter region of protamine 1 (ref. 17) and Tp-1 (ref. 18) has been shown to contain a cAMP response element, but the promoter of MCS has not been well characterized. MCS mRNA is first found in step-3 spermatids in wild-type mice¹⁹. Spermatids developed to this step are still

TABLE 1 Organ weight and hormone analysis in CREM-mutant mice

Measurement	Group	5-week	10-week	15-week
Testis weight (mg)	+/+	159 ± 7	233 ± 12	241 ± 10
	-/-	133 ± 8	140 ± 6	130 ± 8
		<i>P</i> = 0.051	<i>P</i> = 0.000009	<i>P</i> = 0.002
Epididymis weight (mg)	+/+	48 ± 8	79 ± 4	84 ± 7
	-/-	48 ± 4	66 ± 4	72 ± 9
		<i>P</i> = 0.98	<i>P</i> = 0.13	<i>P</i> = 0.34
Seminal vesicle weight (mg)	+/+	53 ± 7	147 ± 16	145 ± 20
	-/-	61 ± 1	108 ± 7	141 ± 23
		<i>P</i> = 0.45	<i>P</i> = 0.04	<i>P</i> = 0.92
Serum testosterone (nmol l ⁻¹)	+/+	5.6 ± 4.2	24.9 ± 6.5	10.8 ± 5.5
	-/-	13.3 ± 8.8	49.3 ± 19.6	43.2 ± 20.6
		<i>P</i> = 0.45	<i>P</i> = 0.25	<i>P</i> = 0.21
Testicular androgens (ng g ⁻¹)	+/+	74 ± 51	123 ± 49	138 ± 105
	-/-	370 ± 190	342 ± 88	499 ± 235
		<i>P</i> = 0.17	<i>P</i> = 0.05	<i>P</i> = 0.50
Serum FSH (μg l ⁻¹)	+/+	ND	35.7 ± 2*	33.1 ± 6.2
	-/-	ND	42.6 ± 2.3*	43.2 ± 7.1
			<i>P</i> = 0.064	<i>P</i> = 0.30

Sample size was 5 mice for 5- and 15-week groups, and 8 for 10-week mice. Blood was obtained from the portal vein. Testosterone and FSH were determined from serum by established double-antibody radioimmunoassays as described previously²³. Significant differences were observed between genotypes of testis weight consistently throughout development (5 to 15 weeks). No decrease in serum testosterone or testicular androgens was observed in the mutant animals. ND indicates measurement was not done owing to insufficient amounts of serum.

* Sample size was 4 mice.

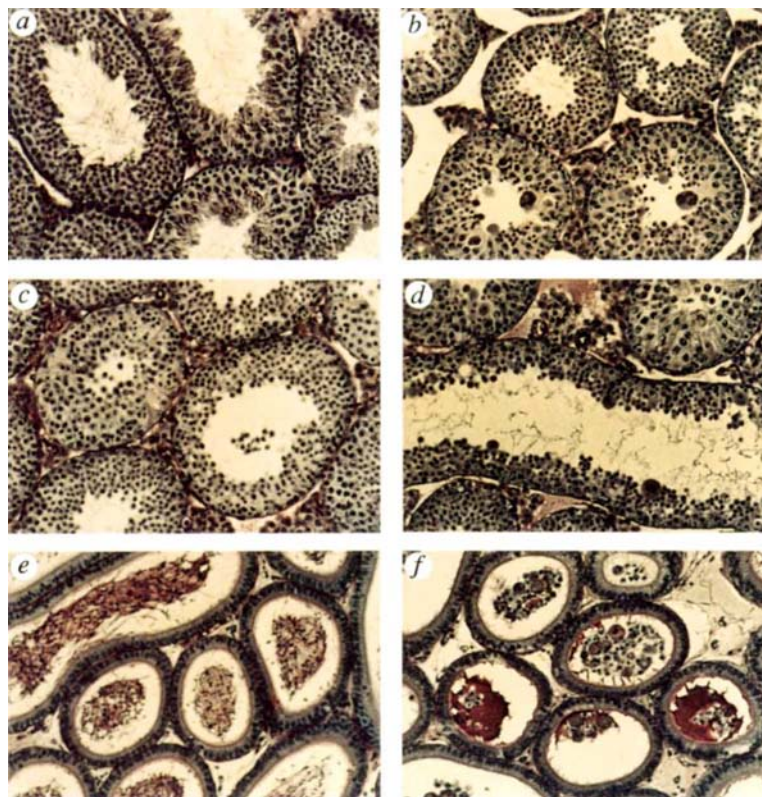


FIG. 2 Testicular (a-d) and epididymal (e-f) sections in wild-type (a, e) and CREM-deficient (b-d, f) mice. Spermatogenesis is complete at 5 weeks, as shown by the abundance of elongating and elongated spermatids (testicular sperm) in all seminiferous tubules of wild-type mice (a). Note the complete absence of spermatid elongation in 5-week-old CREM-mutant mice (b), with some of the round spermatids being released in clusters. A similar picture emerges for the 10-week-old (c) and 15-week-old (d) mutant animals. Spermatids appear to continue to be produced but fail to develop further into testicular spermatozoa. Rather, the round spermatids are eventually detached from the seminiferous epithelium, either individually or in clusters resembling syncytia. No other testicular abnormality was noted.

METHODS. Mice at 5, 10 and 15 weeks of age were killed and tissues removed. Testes were fixed in Bouin's fluid, embedded in Paraplast, cut at 3-5 μm and stained with periodic acid Schiff's base and haematoxylin. Staging of spermatids was according to ref. 26.

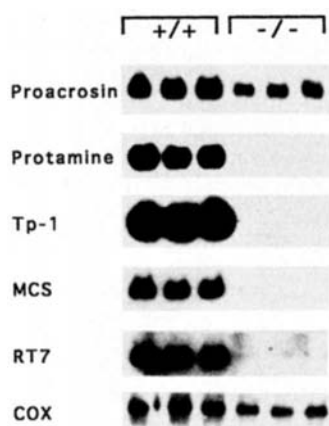


FIG. 3 Expression analysis of genes involved in spermatogenesis. Northern blot analysis with testis-specific probes reveals that several of these mRNAs are absent from CREM-mutant testis. Rehybridization of the filters with cytochrome c oxidase (COX) indicates equal loading.

METHODS. Total RNA was prepared from testis from three 10-week-old CREM $-/-$ mice and their wild-type littermates, as in Fig. 1. Northern blot analysis of 10 μ g RNA, each with probes for mouse proacrosin²⁷, MCS²⁸, probes for both proacrosin and MCS were provided by W. Engel; protamine (obtained from American Type Culture Collection)²⁹, TP-1 (obtained from American Type Culture Collection)³⁰, RT-7 (provided by F. A. van der Hoorn); and COX³¹.

present in the CREM-mutant testis, although to a reduced extent, and so the dramatic reduction of MCS mRNA in homozygous mutant mice seems to be a direct consequence of the CREM mutation.

RT7 is a male germ cell-specific gene that is expressed at a very high level in early spermatids. Putative regulatory elements in its promoter include potential binding sites for members of the CREB/ATF transcription factor family^{20,21}. In testis of homozygous mutant mice, RT7 mRNA is completely absent, indicating that CREM is essential for the activation of the RT7 gene.

Through targeted inactivation of the CREM gene we have demonstrated its importance in male fertility. The identification of a transcription factor that controls several genes involved in spermatogenesis and is directly or indirectly responsible for their activation is an important step in understanding the complex process of spermatogenesis. It seems likely that transcriptional control of these genes would be coordinated with meiosis because during this time transcription factors such as CREM could respond to the changes in chromatin structure that occur during the mitotic and meiotic replications.

About one-third of infertile men fall into the category of idiopathic infertility, that is, they suffer from deficient spermatogenesis even though gonadotropic and androgenic hormone secretion are not subnormal²². The causes underlying this condition have remained unknown, and research in this area has been hampered by the lack of availability of appropriate animal models. Thus the specificity of the CREM mutation means that CREM homozygous mutant mouse could become instrumental in unravelling those mammalian factors pivotal for the postmeiotic differentiation and maturation of haploid germ cells into spermatozoa. It would also provide a potential model for developing a strategy to regulate male fertility. □

Received 30 October; accepted 1 February 1996.

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ACKNOWLEDGEMENTS. We thank A. Kieve-Nebenius, S. Ridder, H. Glaser, A. Rösner, R. Sandhowe, M. Niemeier and E. Langener for technical assistance, and W. Engel for critically reading the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the European Community.

Broadband neural encoding in the cricket cercal sensory system enhanced by stochastic resonance

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SENSORY systems are often required to detect a small amplitude signal embedded in broadband background noise. Traditionally, ambient noise is regarded as detrimental to encoding accuracy. Recently, however, a phenomenon known as stochastic resonance has been described in which, for systems with a nonlinear threshold, increasing the input noise level can actually improve the output signal-to-noise ratio over a limited range of signal and noise strengths. Previous theoretical and experimental studies of stochastic resonance in physical^{1–7} and biological^{6–10} systems have dealt exclusively with single-frequency sine stimuli embedded in a broadband noise background. In the past year it has been shown in a theoretical and modelling study that stochastic resonance can be observed with broadband signals^{11,12}. Here we demonstrate that broadband stochastic resonance is manifest in the peripheral layers of neural processing in a simple sensory system, and that it plays a role over a wide range of biologically relevant stimulus parameters. Further, we quantify the functional significance of the phenomenon within the context of signal processing, using information theory.

The effect of ambient noise on signal encoding was investigated in the cercal system of the cricket *Acheta domestica*, a well characterized mechanosensory system capable of detecting small-amplitude low-frequency air disturbances^{13–18}. Movement of air particles, such as those caused by an approaching predator or conspecific, excite mechanosensory afferents that synapse on

interneurons in the cricket's terminal abdominal ganglion. These interneurons encode information about the velocity and direction of air-current stimuli in their patterns of spikes (action potentials). Intracellular recordings were made from the two types of these identified interneurons having the lowest observed thresholds for air displacement¹⁴. Their responses to a series of air-current stimuli presented at their directions of maximal response were recorded.

To demonstrate stochastic resonance in the conventional manner^{2,6-10}, we presented very low amplitude (just supra-threshold) sinusoidal air-current signals of 23 Hz to the cricket in the presence of varying levels of 5–400 Hz white-noise background (Fig. 1). As has been seen in other systems, the output signal-to-noise ratio (SNR) increased with added input noise to a

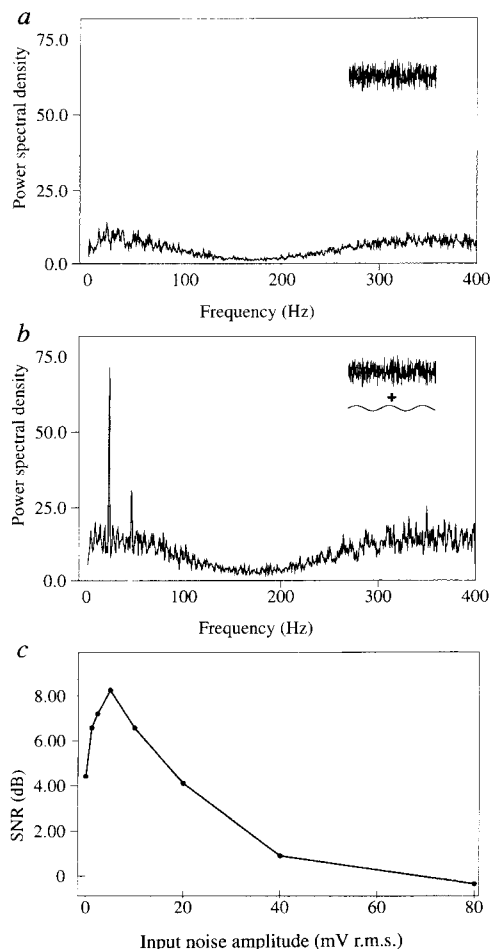


FIG. 1 Results of the standard^{2,6-9} stochastic resonance experimental procedure. Very low amplitude (near threshold) sine-wave stimuli in the region of best sensitivity (23 Hz) were presented with varying levels of broadband (5–400 Hz) white-noise background air disturbances. *a*, Output power spectrum of the spike-train response patterns for the broadband white noise presented alone. Average is over 12 presentations of a 3.2-s segment of white noise. Inset is a portion of the noise waveform. *b*, Output power spectrum for the same white noise as presented above along with a 23-Hz sine wave of r.m.s. amplitude 1/25 that of the noise. Inset is portions of both waveforms, presented simultaneously to the system at the direction of the interneuron's peak response. *c*, Output SNR for varying amplitudes of input noise, calculated by the standard formula^{2,5,7,9}. $SNR = 10 \log(S/N(f))$, where $N(f)$ is the amplitude of the noise power density at the stimulus frequency when presented alone, and S is the area under the signal peak above the noise in the joint presentation case. For the 23 Hz sine wave at this amplitude, a stochastic resonance peak was observed near an added input noise level of 25 times the r.m.s. amplitude of the stimulus.

maximum (the stochastic resonance peak), and then fell as the input noise amplitude was increased beyond that level.

Though previous studies of stochastic resonance have been formulated within the context of the SNR, it is the total information encoded about the signal that is the biologically relevant quantity to consider. We have quantified the encoding accuracy in terms of Shannon's transinformation (or mutual information) rate¹⁹ across the entire range of air-current frequencies and amplitudes to which these particular neurons are known to respond¹³. The test signals were broadband (5–400 Hz) white-noise air-current waveforms, presented at multiple r.m.s. amplitudes. The waveforms presented as additive noise were also 5–400 Hz white-noise air currents, constructed to be uncorrelated with the test-signal waveforms. As the organism has no way of discriminating signal from noise in this, or any other, experiment, white-noise waveforms were chosen for both, to allow the study of information flow in a sensory system. Our approach was to assess the extent to which the additive noise affected the transinforma-

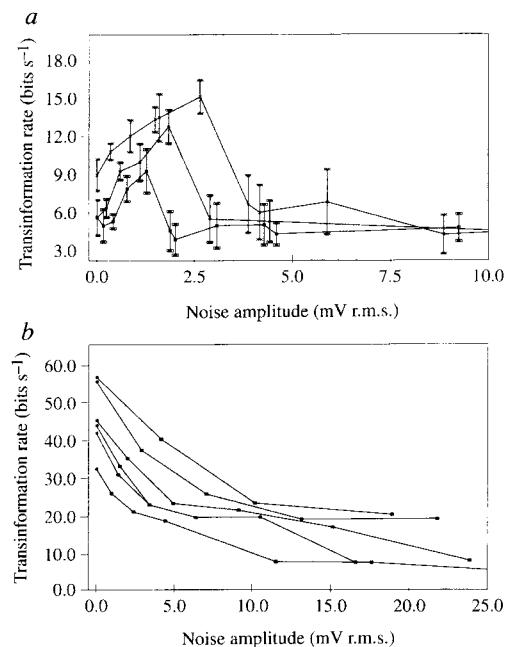


FIG. 2 Results of presentations of broadband stimuli, covering the entire frequency and amplitude sensitivity ranges of the neurons, alone and with varying levels of broadband noise. *a*, Transinformation rates as a function of added input noise amplitude for three low-amplitude signals (~ 0.06 , 0.09 and 0.12 mm s^{-1} r.m.s. velocity, bottom to top curves, respectively). Note that information rates always increased with increasing signal strength, as would be expected. Stochastic resonance peaks are seen for all three. Error bars represent the s.e.m. in the transinformation, calculated by jackknife bootstrapping statistics.^{13,14} *b*, Transinformation rates as a function of noise for six higher amplitude signals (~ 0.33 – 1.5 mm s^{-1} r.m.s. velocity, bottom to top curves respectively). In these cases the neuron was encoding well with no noise, and additional input noise degraded the encoding rate. METHODS. Forty different segments of broadband (5–400 Hz) white noise air-current signals, each 3.2 s in duration, were presented at 17 different mean intensity levels, spanning most of the dynamic range of the interneurons. Each signal was presented either alone or simultaneously with an uncorrelated 5–400 Hz white noise background at one of 17 possible intensities. Several independent white-noise stimuli were used for each stimulus/noise amplitude pair, to eliminate possible stimulus-specific responses. Multiple presentations of each stimulus/noise combination were made at each intensity, and trials were completely randomized with regard to stimulus waveform, stimulus amplitude, noise waveform and noise amplitude. The interstimulus waiting period was 3 s. Total rate of information transmitted about the stimulus waveform by the elicited spike trains is plotted. Note that the information rate is directly related to the SNR through Shannon's formula¹⁹: $R = \int_0^\infty \partial f \log_2(1 + SNR)$ for gaussian distributions of signal and noise, such as those used here.

tion between the test signal and the cell's response. We calculated the coherence between the signal and the elicited spike trains as a function of frequency. This was accomplished by calculating the cross-correlation of the test-signal waveform and the spike trains across all frequencies, and then normalizing the result by the product of their autocorrelations^{13,20,21}. The rate, R , at which information was transmitted by each spike-train response about the corresponding signal was then computed through Shannon's formula for transinformation with independent white noise¹⁹:

$$R = \int_0^\infty \partial f \log_2 \left(\frac{S+N}{N} \right)$$

(where S and N are the powers of the signal and the noise, respectively), and by exploiting the fact that for frequency independent linear stimulus coding, $(S+N)/N = 1/(1-g)$, where g is the coherence function^{13,14}. Coherences between each signal waveform and its corresponding spike train were computed in the presence of several different noise waveforms, each of which were uncorrelated with the signal. Additionally, several signal waveforms were presented at each amplitude, and multiple repetitions of each signal/noise waveform combination were presented. Thus, we ensured that the measure of the accuracy of the neuronal information channel represented signal encoding, and not encoding of the noise background.

For the lowest amplitude decade of the three-decade sensitivity range of these cells, a peak in the transinformation rate was seen at an added noise level of approximately 3.2 times the r.m.s. signal amplitude (Fig. 2a). Theory predicts that such a peak should exist⁴⁻⁸. However, the peak occurred at an almost constant ratio of noise-to-signal input powers across an order of magnitude of

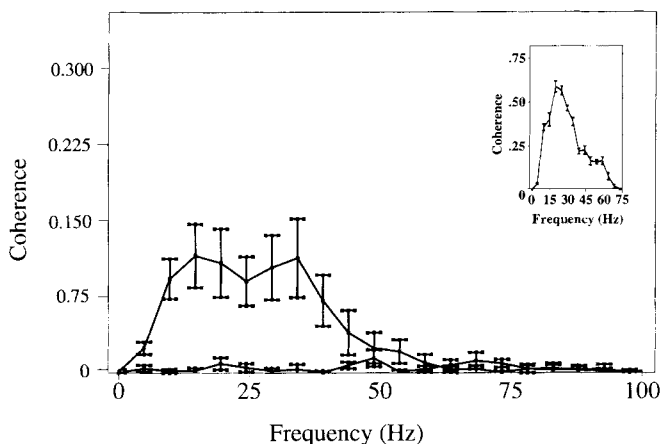


FIG. 3 Coherence of the elicited spike trains with the signal in the presence and absence of background noise, across the frequency range of an example cell. Integrating $1/(1 - \text{coherence})$ over all frequencies gives the transinformation rate (see text) as plotted in Fig. 2. Frequency-dependent coherence is shown for a 5–400 Hz white-noise signal presented at very low amplitude alone (lower line of points) and along with uncorrelated 5–400 Hz white-noise backgrounds with 3.2 times the r.m.s. amplitude of the signal (upper line of points). The plotted points are the averages of multiple presentations of identical signals with several different uncorrelated background noise waveforms. Inset is the coherence of the cell's spiking response to the same 5–400 Hz signal presented at 50 times greater r.m.s. amplitude, where encoding quality is near maximal. Note that an enhancement of the response was evident over a broad frequency sensitivity range of the cell (~5–60 Hz), and that the noise-enhanced coherence function had the same general shape as the high stimulus amplitude curve. That is a broad peak was centered at about 25 Hz, a shoulder was centered at around 50 Hz and the coherence dropped to insignificance by 70 Hz. In general, coherence improvements due to added noise were observed across most or all of the frequency operating range for the majority of the cells analysed. Improvements in coherence of the spike trains with the stimulus cannot simply be the result of additional power in that frequency, as the stimulus and noise were uncorrelated. Thus, the spikes were actually improving their encoding of the signal.

stimulus amplitudes, and not at decreasing noise amplitudes for increasing signal amplitudes. This result was unexpected and we suggest it is a consequence of adaptation. For higher input signal amplitudes, added input noise had a purely detrimental effect on signal encoding (Fig. 2b).

As the signal waveform contained all frequencies from 5 Hz to 400 Hz, the effect of added noise could be evaluated independently at each frequency (Fig. 3). For a neuron sensitive to relatively broadband stimuli, such as the interneurons studied here, stochastic resonance would only be a functionally significant phenomenon if it proved beneficial to signal encoding across a large portion of the frequency range of interest, and not just for the restricted range of frequencies suggested by classical transition-rate theory^{4,5,8}. For the cells studied here, significant improvements in SNR were indeed observed across most, or all, of the range of frequencies to which these neurons showed sensitivity.

We also calculated the average amount of information carried by individual spikes in the presence of different background noise levels, by dividing the calculated transinformation rates by the mean spike rates in each case. Plots of the bits of transinformation per spike against background-noise amplitude for low stimulus amplitudes demonstrated the same general stochastic resonance phenomenon (Fig. 4). Thus, the accuracy of individual spike placement actually increased with certain levels of added input noise.

Considerable improvements in signal encoding were observed in the perithreshold stimulus regime though the addition of external noise. The stochastic resonance-related increases in transinformation rate for broadband signals were as high as 150%, and up to 600% increases in SNR were observed for sinusoidal signals. Although the magnitude of the stochastic resonance effect varied under the wide variety of the signal and noise conditions tested, every cell in our experimental set (14 cells in 12 animals) showed a significant degree of encoding enhancement through stochastic resonance.

Air displacements during the attack of the wasp *Liris niger*, a predator of *A. domestica*, have been reported to be in the same

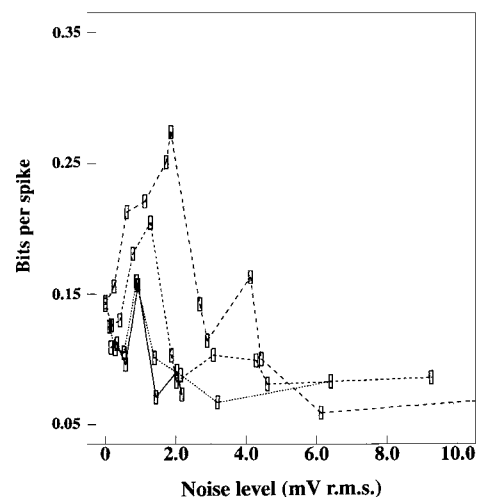


FIG. 4 Average bits of information per spike for four low-amplitude signals as a function of background noise amplitude (~0.04, 0.06, 0.09 and 0.12 mm s⁻¹ r.m.s. velocities, bottom solid curve to top dashed one, respectively). Transinformation rates in each experimental condition were divided by the mean spike rate of the neuron for that condition, giving the average number of bits of information carried per spike. Note that bits per spike always increased with increasing signal strength. Over an entire decade of signal amplitudes, a peak very similar in shape to the standard stochastic resonance peak was seen. Thus, the addition of input noise increased the cells' spiking probability, increased the amount of transmitted information, and also increased the precision with which individual spikes were being placed to encode the stimulus.

frequency range (5–50 Hz), and escape behaviour has been reliably observed for velocities in the range where we observed stochastic resonance effects (below 1 mm s^{-1})²². Escape responses are also reliably evoked for this velocity range from a similar system in the cockroach, where it has been noted that the difference between an escape response and no behaviour was a matter of a few interneuron spikes²³. Clearly, stochastic resonance must be considered as having significance within the context of neural coding and computation. □

Received 28 August 1995; accepted 4 January 1996.

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ACKNOWLEDGEMENTS. We thank F. Theunissen for many useful discussions. This work was supported by a grant from the NIH.

β-Amyloid-mediated vasoactivity and vascular endothelial damage

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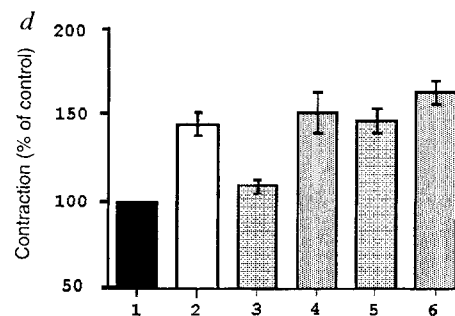
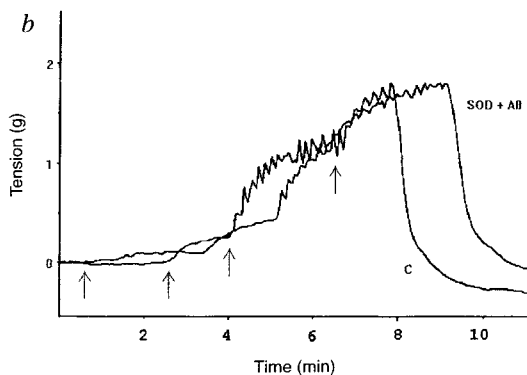
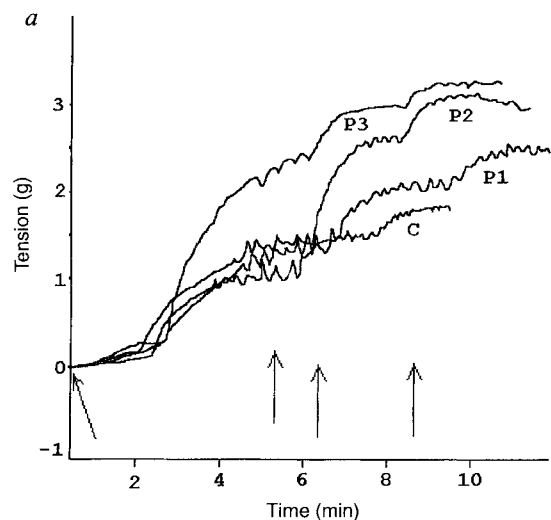
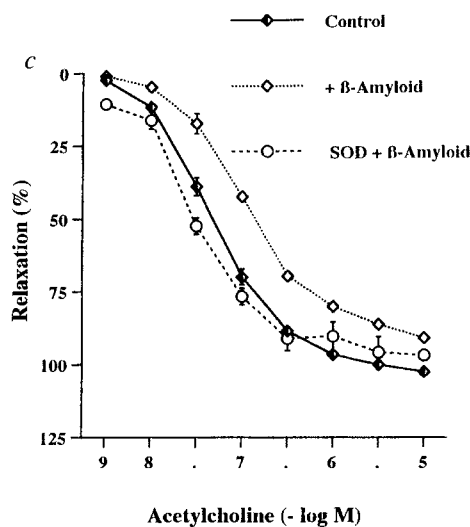
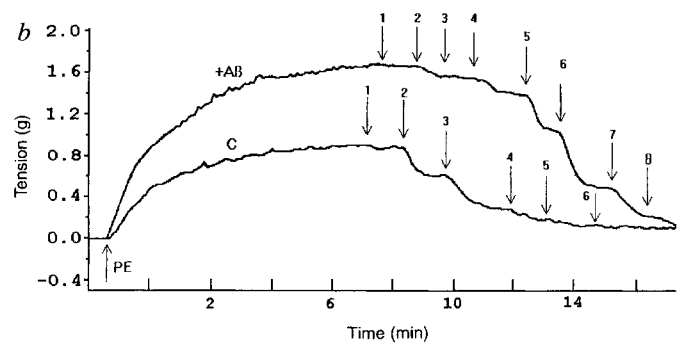
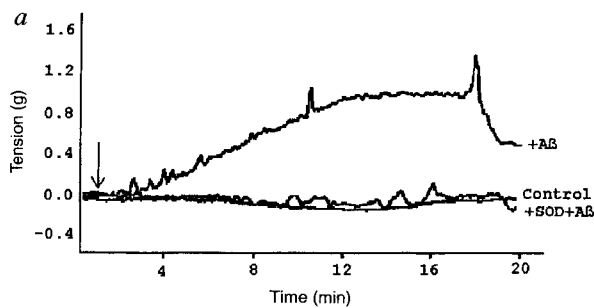
DEPOSITS of β-amyloid are apparent in ageing and Alzheimer's disease¹, but the role of this peptide in neurodegeneration is unclear². The free-radical theory of ageing may also account for Alzheimer-type degeneration and consequently links between free-radical generation and β-amyloid have been sought³. We demonstrate here that β-amyloid interacts with endothelial cells on blood vessels to produce an excess of superoxide radicals, with attendant alterations in endothelial structure and function. The superoxide radical can scavenge endothelium-derived relaxing factor and produce potent oxidizing agents, which can cause lipid peroxidation and other degenerative changes⁴. The alterations in vascular tone and endothelial damage are prevented by the oxygen-radical-scavenging enzyme superoxide dismutase. These observations suggest a normal vasoactive role for β-amyloid as well as a mechanism by which β-amyloid may play a role in vascular abnormalities and neurodegeneration mediated by free radicals.

We examined the relationship between β-amyloid peptides (Aβ 1-39, 1-40, 1-42) and oxygen radical formation in a system known

FIG. 1 a, β-Amyloid-induced contraction of blood vessel with intact endothelium. Freshly excised rat aorta was cut into ring segments 3 mm in length. The tissue was then mounted on stainless steel hooks attached to a force displacement transducer and equilibrated under the optimum tension of 1.3 g in a 10 ml tissue bath containing oxygenated (5% CO₂ in O₂) Krebs buffer at 37 °C. The buffer was changed every 15 min. After incubation for 60 min the viability of the blood vessel with intact endothelium was established by contracting the tissue using PE. Subsequent addition of acetylcholine demonstrated the characteristic relaxation of blood vessel. The vessel preparations were washed 15 min before various manipulations. The endothelium was removed from some tissues by rubbing the inside of the lumen with a spatula for 6–10 s followed by subsequent rinsing with buffer. The removal of endothelium was confirmed by testing for absence of relaxation to acetylcholine as suggested¹⁴. The arrow indicates the addition of 10^{-6} M β-amyloid. The control tissue received no β-amyloid. SOD (150 units ml⁻¹) was added 30 s before the addition of β-amyloid. The maximum contraction induced by β-amyloid was $1.14 \pm 0.13 \text{ (g)}$ and in presence of SOD, the contraction was reduced to $0.04 \pm 0.02 \text{ (g)}$. Values represent the mean $\pm$ s.e.m. of five separate experiments. b, c, Showing the effect on acetylcholine relaxation response of rat aorta after a single dose of PE with and without pretreatment with β-amyloid. b, Trace shows effect of pretreatment with a single dose (10^{-6} M) of β-amyloid on relaxation response. Aorta was precontracted with 10^{-7} M PE and relaxed with increasing doses of acetylcholine as arrowed ($1, 10^{-9} \text{ M}$; $2, 10^{-8} \text{ M}$; $3, 5 \times 10^{-8} \text{ M}$; $4, 10^{-7} \text{ M}$; $5, 5 \times 10^{-7} \text{ M}$; $6, 10^{-6} \text{ M}$; $7, 10^{-5} \text{ M}$; $8, 5 \times 10^{-5} \text{ M}$). c, The acetylcholine relaxation curve is shifted to the right but maintains its sigmoidal form, indicating attenuation of acetylcholine-induced relaxation. The relaxation by acetylcholine is shown under control conditions and following 15 min incubation with 10^{-6} M β-amyloid protein. The aorta was precontracted submaximally with 10^{-7} M PE. The concentration of acetylcholine used was 10^{-9} M to 10^{-5} M . Values represent the mean $\pm$ s.e.m. of five or more experiments. The cumulative percentage relaxation is statistically significantly lowered by β-amyloid protein at all points after 10^{-8} M acetylcholine ($P < 0.05$ or less). β-amyloid pretreatment opposes the effects of lower doses of acetylcholine. At higher doses of acetylcholine greater percentage changes in relaxation occur, preserving the sigmoidal response. Pretreatment of the blood vessel with SOD (150 units ml⁻¹) 30 s before the addition of β-amyloid blocked the attenuation of acetylcholine-induced relaxation. The relaxation induced after pretreatment with SOD + β-amyloid was significantly greater than that produced in vessels pretreated with β-amyloid alone at acetylcholine doses of 10^{-8} M or greater ($P < 0.05$ or less).

FIG. 2 a, Trace shows effect of β-amyloid on contraction and relaxation of blood vessel with intact endothelium. Control contraction with increasing concentrations of PE (2, 4, 8 and $16 \times 10^{-8} \text{ M}$) is shown by trace c. The procedure was then repeated after incubation of the tissue with 10^{-6} M β-amyloid for 15 min (P1). The tissue was then rinsed and contractions repeated twice after 15 min incubation (P2 and P3) without further addition of β-amyloid. Arrows are shown for points of addition for P3. Control incubations with buffer alone showed no increase with repeated contractions. b, Representative figure showing the effect of SOD on β-amyloid (10^{-6} M) augmentation of PE-induced contraction. The control curve without β-amyloid is shown (C). SOD was added 30 s before the addition of 10^{-6} M β-amyloid. Arrows represent the points of addition of PE for the control. c, The effect of preincubation of blood vessel with 10^{-6} M β-amyloid on contraction induced by phenylephrine (2, 4, 8 and $16 \times 10^{-8} \text{ M}$). The maximum contraction in the absence of β-amyloid is taken as 100% and the values obtained after exposure to β-amyloid are expressed as per cent of control value. After washing out PE and acetylcholine from the control, β-amyloid augmented the PE-induced contractions (P1). This effect was blocked by SOD. Washing out the PE and acetylcholine of the post-peptide contraction and repeating the contractions caused further enhancement (P2). Values represent the mean $\pm$ s.e.m. of nine or more experiments. The difference between control and P1 was significant for increasing doses of PE ($P = \text{NS}$, < 0.005 , < 0.005 , < 0.001 , respectively) and differences between β-amyloid treated and SOD plus β-amyloid treated were also significantly different with increasing doses of PE ($P = \text{NS}$, < 0.005 , < 0.005 , < 0.001 , respectively) indicating reduction of β-amyloid enhancement by SOD. d, Specificity of SOD action is illustrated by the use of active and inactive preparations of SOD. The aortic rings were preincubated with the appropriate test compounds before the addition of 10^{-6} M β-amyloid. After 15 min incubation

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(c) Effect of preincubation on contraction

PE ($\times 10^{-8}$ M)	Control	+ β -amyloid P1	+ β -amyloid P2	+ β -amyloid + SOD
2.0	18.0 (8.0)	13.3 (5.9)	20.0 (5.0)	12.0 (5.7)
4.0	65.0 (5.8)	75.0 (6.0)	99.0 (15.0)	62.0 (11.0)
8.0	86.0 (4.0)	105.0 (15.0)	133.0 (20.0)	93.0 (13.0)
16.0	100.0 (0.0)	128.0 (11.7)	141.0 (17.6)	110.0 (11.0)

the preparations were contracted with 10^{-7} M PE as described earlier. Control contractions with PE alone were carried out in the presence of various test compounds. Values represent the mean $\pm$ s.e.m. of four or more experiments. The heat-inactivated SOD was prepared by keeping in boiling water for 30 min followed by cooling before use. The hydrogen peroxide-inactivated SOD was prepared by exposing SOD to 10 mM H_2O_2 for

30 min at pH 8.5, and subsequent separation using a Microcon filtration system. The amyloid-induced PE contraction was blocked by active SOD. Neither albumin nor inactivated SOD had any effect on the amyloid activity. Bars: 1, control; 2, + β protein; 3, active SOD + β protein; 4, albumin + β protein; 5, heat-inactivated SOD + β protein; 6, H_2O_2 -inactivated SOD + β protein.

to respond physiologically to free radicals: endothelium-mediated vasoactivity⁵. β -Amyloid constricted the blood vessel and the vessel was sensitive to β -amyloid in the concentration range $0.25\text{--}2.0 \times 10^{-6}$ M (Fig. 1a). The vasoactive property of β -amyloid occurred only in the presence of endothelium, suggesting that this action is mediated through the endothelium. The β -amyloid-induced vasoactivity had the following characteristics. The blood vessel showed an immediate response and vasoactivity was evident within 30 s. The initial detectable change was a minor relaxation <0.1 g (tension). This was followed by a series of contractions and relaxations. There were periodic bursts of contractions followed by immediate relaxations (Fig. 1a). This activity persisted for a period of 15–20 min. To investigate the role of oxygen radicals in mediating the vasoactivity elicited by β -amyloid, the superoxide scavenging enzyme superoxide dismutase (SOD) was added 30 s before the addition of β -amyloid. Pretreatment with SOD eliminated the vasoactivity induced by β -amyloid, suggesting that the vasoactive action of β -amyloid involves the formation of superoxide radicals. If SOD was added after incubation with β -amyloid, the vasoactivity was not abolished. Washing the tissue and removing the SOD without further addition of β -amyloid produced changes characteristic of endothelial dysfunction: enhanced vasoconstriction and diminished vasodilation. Thus there is a continuous requirement for SOD to prevent the effects of β -amyloid. To ensure continued availability of SOD it was necessary to include SOD in the buffer used to rinse the tissue in the SOD-treated preparation.

Pretreatment with β -amyloid significantly reduced relaxation induced by the vasodilator acetylcholine (Fig. 1b, c). Washing the tissue after β -amyloid treatment did not restore the acetylcholine-induced relaxation to control levels, indicating that β -amyloid had altered endothelial function. The acetylcholine-resistant relaxation was overcome by the nitric oxide (NO) donor sodium nitroprusside (data not shown). Addition of SOD slightly potentiated the relaxation response to acetylcholine and at the same time antagonized the effect of β -amyloid.

Figure 2 shows the enhancement of contraction induced by the vasoconstrictor phenylephrine (PE) after β -amyloid treatment. Pretreatment with the enzyme SOD almost totally removed the enhancement of contraction by β -amyloid (Fig. 2b, c). Washing

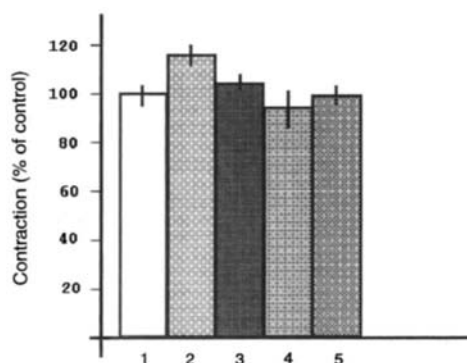


FIG. 3 Effect of various drug treatments on β -amyloid augmentation of PE-induced contractions. Blood vessel rings were initially contracted with PE (4×10^{-8} M). The tissue was then incubated for 15 min with β -amyloid (5×10^{-7} M) with or without the drug, followed by contractions with PE. The concentrations of compounds used were: L-NMMA, an inhibitor of nitric oxide synthase (10^{-4} M); indomethacin, an inhibitor of the cyclooxygenase pathway (10^{-5} M); SQ 29548, a thromboxane A_2 receptor antagonist (10^{-5} M); and catalase (1,000 units ml^{-1}). Values are represented as percentage of the maximal contraction obtained with β -amyloid and PE alone and standardized to remove the intrinsic effects (if any) of each drug on PE contraction alone. The results represent the mean $\pm$ s.e.m. of five or more experiments. None of the compounds exerted any significant effect on the β -amyloid-induced enhancement of PE-mediated contraction. Bars: 1, β -amyloid + PE; 2, L-NMMA + β -amyloid + PE; 3, catalase + β -amyloid + PE; 4, SQ29548 + β -amyloid + PE; 5, SQ29548 + β -amyloid + PE.

the tissue after treatment with β -amyloid and reapplying PE produced further enhancement of contraction, indicating additional endothelial dysfunction (Fig. 2a, c). Figure 2d shows that heat-inactivated SOD, H_2O_2 -inactivated SOD and the control protein albumin could not protect the blood vessel against the effects of β -amyloid, suggesting that the protective effects were not due to nonspecific binding of β -amyloid by SOD. Removing the SOD by rinsing allows the endothelial dysfunction in tissue pretreated with β -amyloid. This observation also argues against protection by SOD due to binding of β -amyloid, as both would be washed out during rinsing. Proteolytic destruction of β -amyloid by contaminants in the SOD preparation is excluded by the trace

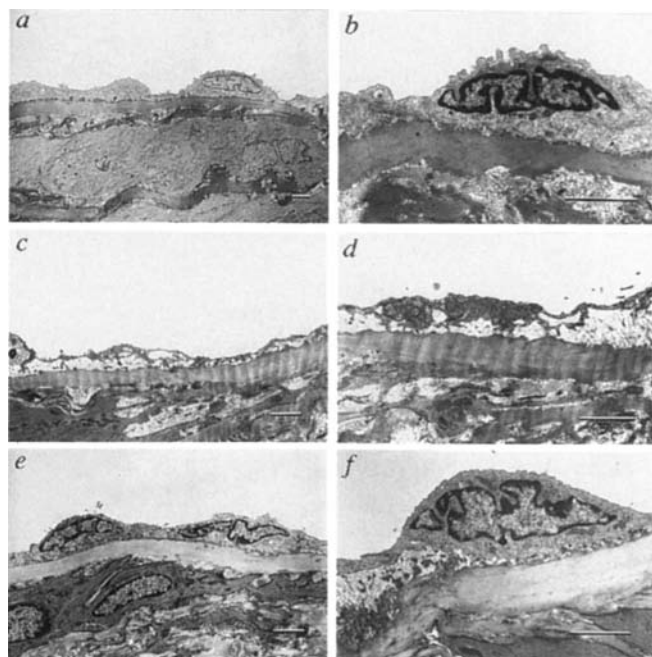


FIG. 4 Electron micrographs showing the endothelial cell layer in aortic rings. Blood vessel rings were incubated as previously described at 37°C for 30 min either alone or in the presence of β -amyloid (10^{-6} M), or SOD (150 units ml^{-1}) plus β -amyloid. The tissue samples were kept in glutaraldehyde solution at 4°C until processed and then rinsed with cacodylate buffer. The tissues were postfixed with 1% buffered osmium tetroxide, dehydrated with graded ethanol solutions, and embedded in a mixture of Epon-Araldite. Ultra-thin sections were stained with uranyl acetate and lead citrate and viewed on a model 7000 Hitachi transmission electron microscope. The inset bar indicates $2\mu\text{m}$. The experiment was repeated three or more times with blood vessels from different animals. a, Control aorta (magnification $\times 2,400$). Three normal endothelial cells are visible closely adhering to the intimal elastic lamina lining the lumen, one sectioned showing the nucleus while two missed the nuclear area in this section. b, Control aorta (magnification $\times 9,500$) showing an intact endothelial cell adhering to the internal elastic lamina. Organelles clearly visible in the cytoplasm include the nucleus, mitochondria and Golgi apparatus. A tight junction can be seen between the endothelial cell and adjoining cell. c, Aortic ring treated with β -amyloid (magnification $\times 4,000$). Visible features include derangement of the endothelial cell layer, a condensed nucleus to the left, separation from the internal elastic lamina and, in areas, denuded internal elastic lamina. d, Aortic ring treated with β -amyloid (magnification $\times 7,000$). At this magnification endothelial damage is clearly visible with cytoplasm and membrane destruction as well as swollen mitochondria and no other recognizable organelle. e, Aorta pretreated with SOD before the addition of β -amyloid (magnification $\times 4,000$). Two intact endothelial cells closely adhering to the internal elastic lamina are observed. f, Aortic ring pretreated with SOD and then treated with β -amyloid (magnification $\times 8,000$). This higher magnification reveals an endothelial cell with normal features adhering to the internal elastic lamina close to a fenestration. Intact organelles such as nucleus, mitochondria and rough endoplasmic reticulum are observed. A tight junction can also be seen with the adjacent cell.

amounts (0.00014%) of protease in the SOD preparation (from Alexis Corporation).

In order to elucidate the mechanism underlying these endothelial changes, various compounds known to affect the activity of oxygen radicals were examined. Under the conditions used here none of the compounds used (L-NMMA, indomethacin, SQ29548 or catalase) had any significant effect on the enhancement response (Fig. 3).

Electron microscopic examination of the blood vessel ring treated under the same conditions (Fig. 4) shows endothelial damage by β -amyloid which is prevented by pretreatment with SOD. Incubation with β -amyloid induces significant damage to the endothelial cells with visible changes in the cell membrane, cytoplasm, nucleus and other organelles. Incubation periods of 15 min and β -amyloid concentrations of $\geq 5 \times 10^{-7}$ M were required to induce the morphological changes observed here. The endothelial damage induced by lower doses of amyloid were also blocked by pretreatment with SOD (150 units ml^{-1}).

In Alzheimer's disease deposits of amyloid β -peptides are seen in the brain^{1,2} and cerebral blood vessels⁶. Abnormalities in microvasculature have been shown to precede other neuropathological features of Alzheimer's disease⁷. Expression of one or more copies of the apolipoprotein $\epsilon 4$ allele of the apolipoprotein E gene confers increased risk for vascular degenerative disease⁸ and also increases the risk for early expression of Alzheimer's disease. The cytotoxic effects of oxygen radicals have been invoked as a major contributor to the pathology of neurodegenerative diseases such as Alzheimer's disease. Much evidence points to the central nervous system microvasculature as the major target of free-radical reaction, and the principal mediator of this damage is probably the superoxide radical⁹. A damaged endothelium will exhibit enhanced vasoconstriction and diminished vasodilation due to impaired availability of endothelium-derived relaxing factor. Endothelial damage is currently recognized as an early event in the development of vascular disease. By providing evidence for direct action of β -amyloid on blood vessels and probable mediation of the effect by oxygen radicals, our findings point to a disruption of vascular function in neurodegenerative diseases. The β -amyloid-mediated endothelial damage occurs at much lower concentrations than those used in previous studies (10–80 μM) showing effects such as alteration of Ca^{2+} homeostasis or indirect toxicity¹⁰, but similar to those showing direct trophic or toxic responses¹¹ or inhibition of potassium channels¹². However, in reports of neurotrophic and toxic effects of β -amyloid, incubation periods of 2–4 days using preaggregated β -amyloid are often required to demonstrate an effect. We demonstrate the action of β -amyloid in less than 1 min without the need for preaggregation, suggesting a new and possibly physiologically relevant effect. In addition the biological response is demonstrated in intact tissue with viable permeability barrier and cell-to-cell interaction. Even though the action of β -amyloid delineated here is on a peripheral blood vessel, such an effect on brain vasculature is likely. If brain- or platelet-derived β -amyloid were to come into contact with the endothelium, the free radical production could induce local damage and reduce local blood flow, further increasing oxidative stress and upregulating neuronal β -amyloid production¹³. Other factors that affect the local oxidative stress on cerebral vasculature would influence this process. The β -amyloid-mediated free-radical production and subsequent endothelial damage may have relevance in head injury/brain oedema and cerebrovascular disease and may play a central role in the Alzheimer's disease process. □

Received 26 September 1995; accepted 10 January 1996.

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ACKNOWLEDGEMENT. This work was supported by the Robert Roskamp Endowed Chair in Biological Psychiatry of which M.M. is the recipient.

Impaired migration but not differentiation of haematopoietic stem cells in the absence of β_1 integrins

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ADHESIVE interactions mediated by integrins of the β_1 subfamily¹ are thought to be critical in controlling differentiation and migration of blood cell precursors^{2–7}. Here we report that chimaeric mice generated with β_1 -integrin-deficient embryonic stem (ES) cells^{8,9} lack $\beta_1^{-/-}$ cells in blood and in haematopoietic organs such as spleen, thymus and bone marrow. Chimaeric embryos contain β_1 -null haematopoietic cells in the yolk sac and in fetal blood but not in fetal liver. We show that such $\beta_1^{-/-}$ haematopoietic stem cells derived from yolk sac of 10.5-day-old chimaeric embryos readily generate erythroid and myeloid colonies and that $\beta_1^{-/-}$ ES cells can differentiate into mature B lymphocytes *in vitro*. Our results indicate that haematopoietic stem cells lacking β_1 integrins can form and differentiate into different lineages but cannot colonize the fetal liver.

During vertebrate embryogenesis, common progenitors of haematopoietic and endothelial cells, called haemangioblasts, develop from extraembryonic mesenchyme¹⁰. Clusters of these cells form blood islands in which haematopoietic precursors are surrounded by endothelium¹¹. In the mouse, migrating haematopoietic stem cells (HSC) colonize the liver¹² at around embryonic day 10. The liver then develops into the major haematopoietic organ until birth.

In this study we investigate the role of β_1 integrins during the differentiation and migration of HSC. We have previously shown that in chimaeric mice ($\beta_1^{-/-} \rightarrow$ wild-type (WT) chimaeras), β_1 integrin-deficient cells are detectable in several organs but never in liver and spleen⁹. We therefore assayed for isoenzymes of glucose-6-phosphate isomerase (GPI) in the haematopoietic organs of six $\beta_1^{-/-} \rightarrow$ WT chimaeras (6–8 weeks old). $\beta_1^{-/-}$ cells were absent not only from spleen but also from thymus, bone marrow (Fig. 1a) and blood (not shown). To exclude the possibility that this absence of $\beta_1^{-/-}$ lymphocytes was due to homing competition by wild-type lymphocytes, we tested the ability of $\beta_1^{-/-}$ ES cells to complement the phenotype of RAG-2(recombinase activating gene-2)-deficient mice upon injection into RAG-2^{-/-} blastocysts ($\beta_1^{-/-} \rightarrow$ RAG-2^{-/-} chimaeras) as described previously¹³. The lack of RAG-2 prevents the rearrangement of lymphocyte antigen receptors and leads to an early arrest in the development of T and B lymphocytes. Therefore $\beta_1^{-/-} \rightarrow$ RAG-2^{-/-} chimaeric mice provide a sensitive system for testing whether $\beta_1^{-/-}$ HSC can generate mature lymphoid cells. The extent of $\beta_1^{-/-}$

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null cell contribution to various tissues was similar in adult RAG-2^{-/-} chimaeric mice (data not shown) and in adult wild-type chimaeric mice⁹. In addition, GPI analysis of haematopoietic organs from six $\beta_1^{-/-}$ → RAG-2^{-/-} chimaeric mice (6–8 weeks old) revealed the absence of β_1 -null cells, apart from one thymus in which mutant cells had probably contributed to the stromal compartment (Fig. 1a). To exclude any possible minor contribution of 129-derived cells to the lymphoid compartment, we performed a detailed flow cytometric analysis of cell populations derived from the lymphopoietic organs of $\beta_1^{-/-}$ → RAG-2^{-/-} chimaeras and of control animals obtained by injecting $\beta_1^{+/+}$ ES clone⁸. Control $\beta_1^{-/-}$ → RAG-2^{-/-} or $\beta_1^{+/+}$ → WT chimaeras, with a coat colour contribution comparable to $\beta_1^{-/-}$ → RAG-2^{-/-} (< 25%), contained large numbers of 129-derived lymphocytes (see below; and data not shown). RAG-2-deficient mice reconstituted with $\beta_1^{+/+}$ cells displayed a normal distribution of T lymphocytes both in thymus and spleen, as assessed by expression of CD4 and CD8 surface markers (Fig. 2a). Surface-IgM-positive B cells were also present in normal numbers in the spleens and bone marrow of these animals (not shown). Indeed, the $\beta_1^{-/-}$ → RAG-2^{-/-} mice contained normal levels of serum IgM (Fig. 2b), and the block in B-cell differentiation at the stage of B220^{low}CD43⁺ pre B-lymphocytes caused by the RAG-2 mutation was overcome in the bone marrow of these animals, which contained normal numbers of B220⁺CD43⁺ cells (Fig. 2a). In contrast, the frequency and distribution of lymphoid cells in the immune organs of $\beta_1^{-/-}$ → RAG-2^{-/-} chimaeric mice were indistinguishable from those of RAG-2^{-/-} mutant mice (Fig. 2a). In

the five mice analysed, we could detect only early thymocytic precursors that were negative for surface CD4 and CD8 (Fig. 2a, upper panel). As in RAG-2 deficient thymuses, these cells expressed high levels of the α -chain of the receptor for interleukin-2 (IL-2R α , CD25), which is characteristic of the major subset of double-negative pre-T-lymphocytes (not shown). In the spleen of these animals, no mature T or B lymphocytes were found (Fig. 2a, and results not shown); in the bone marrow, the differentiation of B lymphocytes was arrested at the same stage (B220^{low}CD43⁺) as in RAG-2^{-/-} mice (see boxed regions in the bone marrow blots of Fig. 2a). In addition, the blood serum of $\beta_1^{-/-}$ → RAG-2^{-/-} chimaeric mice was devoid of IgM (Fig. 2b), thereby excluding the presence of minute amounts of $\beta_1^{-/-}$ B lymphocytes that might have gone unnoticed in the FACS experiment.

We next investigated whether the lack of blood cells in adult chimaeric animals was due to impaired development and/or differentiation of haematopoietic stem cells. First, we examined yolk-sac tissue from 9.5-, 10.5- and 11.5-day-old chimaeric embryos ($\beta_1^{+/+}$ → WT) for the presence of β_1 -null blood islands. Surprisingly, all yolk-sac tissues contained blood islands that expressed β -galactosidase (Fig. 3a). The ability of β_1 -null but not wild-type cells to express this reporter gene⁹ indicates that haematopoietic stem cells develop in the absence of β_1 integrin. Second, we investigated whether β_1 -null haematopoietic precursor cells can differentiate into the various blood cell lineages. Cells were isolated from yolk-sac tissue of 10.5-day-old $\beta_1^{-/-}$ → WT chimaeras and cultured in a semisolid medium containing IL-3 and erythropoietin. We took advantage of the ability of $\beta_1^{-/-}$ cells

FIG. 1 Analysis of the contribution of $\beta_1^{-/-}$ ES cells to haematopoietic organs and blood cells of chimaeric animals by detection of GPI isoenzymes. Control samples of GPI-A and GPI-B isoenzymes were obtained from wild-type D3 ES cells (129) and from wild-type C57BL6 mice (C57), respectively. RAG-2^{-/-} animals express the GPI-C isoform. a, GPI analysis of thymus, spleen and bone marrow of $\beta_1^{-/-}$ → WT, $\beta_1^{-/-}$ → WT and $\beta_1^{-/-}$ → RAG-2^{-/-} adult chimaeras. Two $\beta_1^{-/-}$ → WT chimaeras express GPI-A and GPI-B isoforms, whereas two $\beta_1^{-/-}$ → WT and two $\beta_1^{-/-}$ → RAG-2^{-/-} chimaeras express only GPI-B or GPI-C, respectively (with the exception of one thymus). b, GPI pattern of haematopoietic cells derived from yolk sac of 10.5-day $\beta_1^{-/-}$ → WT chimaeric embryos. Colonies grown in the presence of G418 express the GPI-A isoform. Without G418 selection, only wild-type colonies expressing GPI-B were detected. c, GPI analysis of blood of 13.5-, 14.5- and 15.5-day-old $\beta_1^{-/-}$ → WT chimaeric embryos. Lanes from left to right: control sample, (C57-129) plus three blood samples each from 13.5-, 14.5- and 15.5-day-old chimaeric embryos, respectively. ES-cell-derived GPI-A isoform is detectable in all blood samples in various degrees. d, GPI analysis of liver derived from the same embryos shown in c. ES-cell-derived GPI-A isoform could not be detected in any sample. e, GPI pattern of haematopoietic colonies generated by culturing blood cells (left) or liver cells (right) from 14.5-day chimaeric embryos. Colonies from blood and liver were grown with (G418⁺) or without (G418⁻) G418. Blood cultures gave rise to $\beta_1^{-/-}$ colonies expressing the GPI-A isoform both in the presence and absence of G418. Liver cultures generated colonies only in the absence of G418.

METHODS. GPI analysis has been described⁹. Yolk sac, fetal liver or fetal blood haematopoietic cells were cultured as described^{21,22} in semisolid medium containing erythropoietin (2 U ml⁻¹; Boehringer) and IL-3 (100 U ml⁻¹; Boehringer). For yolk sac stem-cell culture, 5 independent experiments were done pooling yolk sacs of 5 to 10 chimaeric embryos. β_1 -integrin-deficient cells were selected in the presence of 400 μ g ml⁻¹ G418. Ten colonies were collected at day 7 and 14 of incubation and pooled for GPI analysis. In 18 independent experiments, cells from blood and liver of same embryos were cultured and analysed.

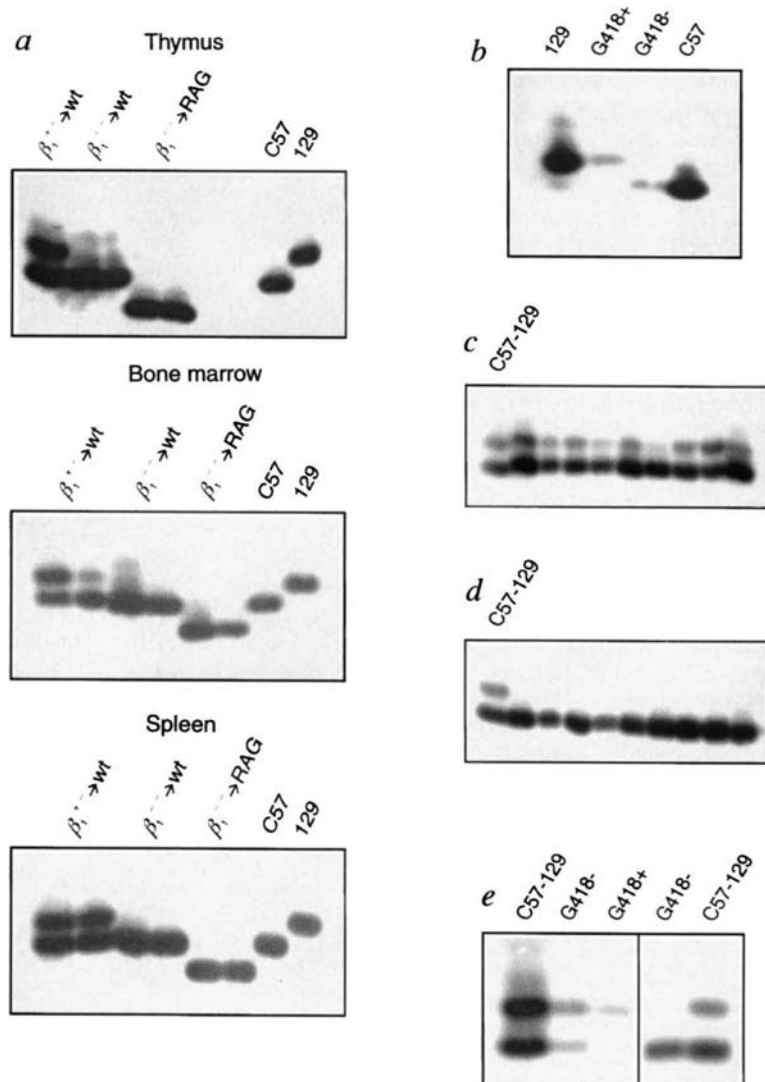
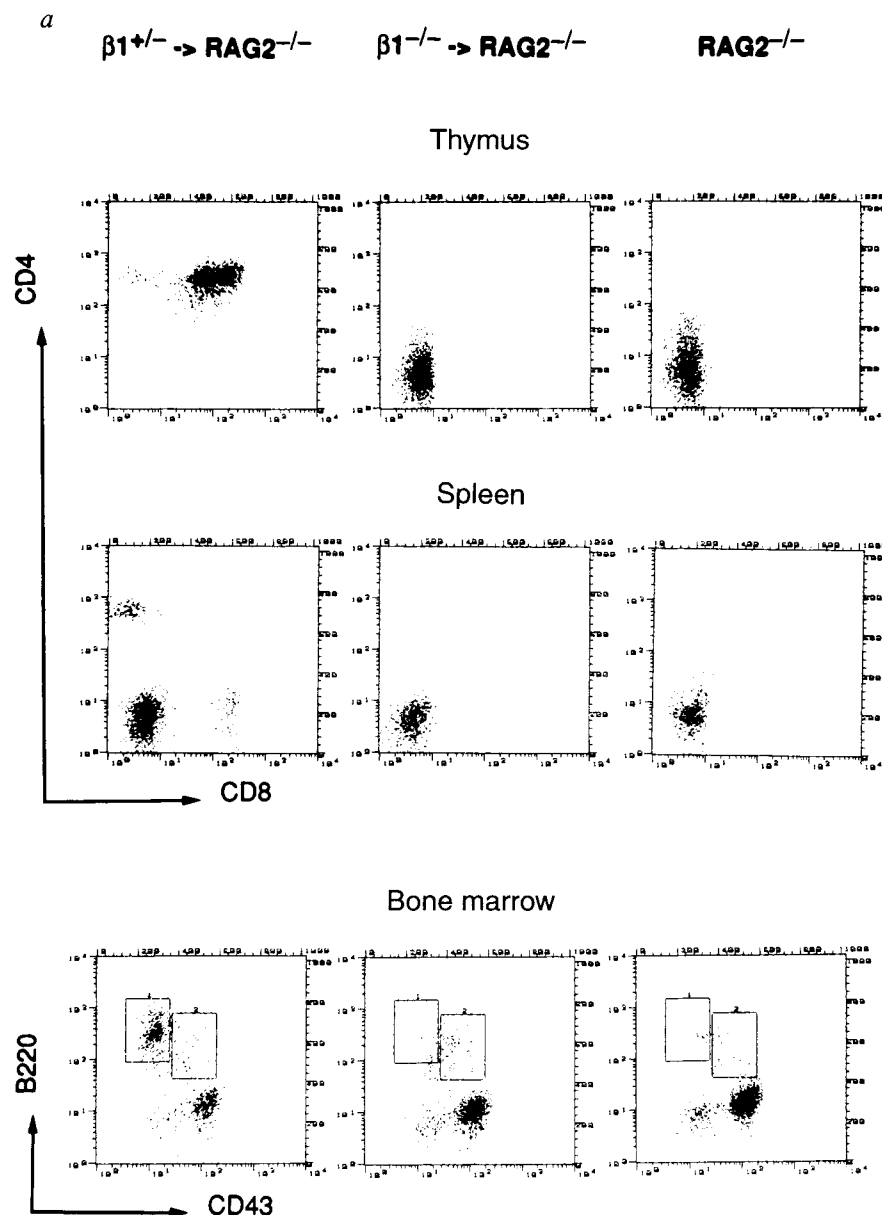


FIG. 2 *a*, FACS analysis of lymphocytes in RAG-2-deficient mice. Staining profiles with the indicated antibodies in the lymphoid gating of the different organs are shown for $\beta_1^{+/-} \rightarrow$ RAG-2 $^{-/-}$ (left), $\beta_1^{-/-} \rightarrow$ RAG-2 $^{-/-}$ (middle) and RAG-2 $^{-/-}$ (right) mice. *b*, Serum IgM levels in wild-type C57BL/6 mice (triangles), $\beta_1^{-/-} \rightarrow$ RAG-2 $^{-/-}$ (squares; 6 animals) or $\beta_1^{+/-} \rightarrow$ RAG-2 $^{-/-}$ (circles; 6 animals) chimaeric mice were measured in an ELISA assay. Average values and standard deviations are shown for chimaeric animals. Serial dilutions of blood serum were incubated in plastic wells coated with monoclonal antibody 1M41 (specific for mouse μ chains²³) and the bound material was then revealed with alkaline-phosphatase-labelled 1M41 antibodies. METHODS. Single-cell suspensions of the indicated lymphoid organs were prepared, stained and analysed as described²⁴ on a FACScan analyser (Becton Dickinson). Cells stained with the indicated antibodies within the lymphoid gating are shown. Representative results from 4 independent experiments are shown. Antibodies used were: phycoerythrin-labelled anti-mouse CD4 and FITC-labelled anti-mouse CD8 (Southern Biotechnology); phycoerythrin-labelled anti-mouse B220 and FITC-labelled anti-mouse CD43 (Pharmingen).



to express the neomycin-resistance gene, introduced with the β_1 gene mutation⁸. To our surprise, after a period of 7–14 days, all colonies grown in G418 expressed the 129 GPI isoform (Fig. 1*b*) and did not express β_1 integrin (Fig. 3*h*), indicating that β_1 -null HSC can form blood cell colonies *in vitro*. The number of blood cell colonies in G418-containing cultures was at least 10-fold lower than in cultures without G418, indicating that the effect of chimaerism on yolk-sac tissues is similar to that in the proper embryo (at day 9.5), where it never exceeds 25 per cent⁹. On the other hand, the cell content of individual colonies was not affected by the absence of β_1 integrin, suggesting that β_1 integrins are not required for blood cell proliferation. Microscopic analysis of cells derived from β_1 -null colonies showed erythroblasts, granulocytes, monocytes and macrophages (Fig. 3*b–f*). To test whether $\beta_1^{-/-}$ lymphoid cells might be generated at a frequency too low to be detected, β_1 -null ES cells were differentiated into embryoid bodies under culture conditions that favour lymphocyte differentiation¹⁴. We found that B lymphocytes expressed the recombinase activating genes RAG-1 and RAG-2 (data not shown) and both *VH/DJH* and *VK/JK* rearrangements in the immunoglobulin heavy- and light-chain genes, respectively (Fig. 4*a*). Furthermore, the frequencies at which $\beta_1^{-/-}$ B lymphocytes developed was the same in wild-type or $\beta_1^{+/-}$ cultures (Fig. 4*b*).

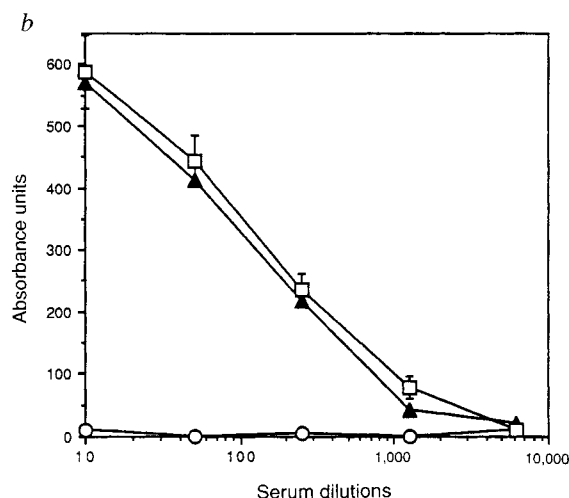


FIG. 3 *In vitro* differentiation of $\beta_1^{-/-}$ haematopoietic progenitors derived from 10.5-day-old yolk-sac tissue. *a*, Whole-mount LacZ staining of yolk sac derived from a 10.5-day post-coitum $\beta_1^{-/-} \rightarrow$ WT chimaeric embryo. The yolk-sac wall contains β -galactosidase-expressing $\beta_1^{-/-}$ blood islands (arrows). *b-f*, Giemsa staining of $\beta_1^{-/-}$ blood cells obtained after *in vitro* differentiation of yolk-sac-derived haematopoietic progenitors. *b*, Erythroid colony showing cells at various stages of differentiation, including proerythroblasts and erythroblasts. *c*, Erythroblast. *d*, Neutrophil precursor (arrow). *e*, Macrophage. *f*, Monocyte. *g, h*, Immunostaining of normal (*g*) and mutant (*h*) macrophages with anti-mouse β_1 integrin antibodies. Scale bars, 100 μ m in *a*, 10 μ m in *b-h*.

METHODS. For staging of chimaeric embryos, noon of the day of formation of the vaginal plug in the foster mother was considered to be day 0.5. Fixation and LacZ-staining of whole-mount specimens obtained from 9.5-, 10.5- and 11.5-day-old $\beta_1^{-/-} \rightarrow$ WT chimaeras were done as described⁹. For Giemsa staining, cells were collected at day 14 of incubation in G418-containing culture medium (as for Fig. 1). For immunocytochemistry (*g, h*) cells were fixed in 4% paraformaldehyde, washed in PBS and incubated for 1 h at room temperature with anti- β_1 purified rabbit IgG²⁵ diluted to 15 μ g ml⁻¹ in PBS containing 5% mouse serum. Cells were then washed in PBS and incubated with anti-rabbit IgG Cy3 conjugate (Jackson ImmunoResearch) diluted according to the manufacturer's instructions.

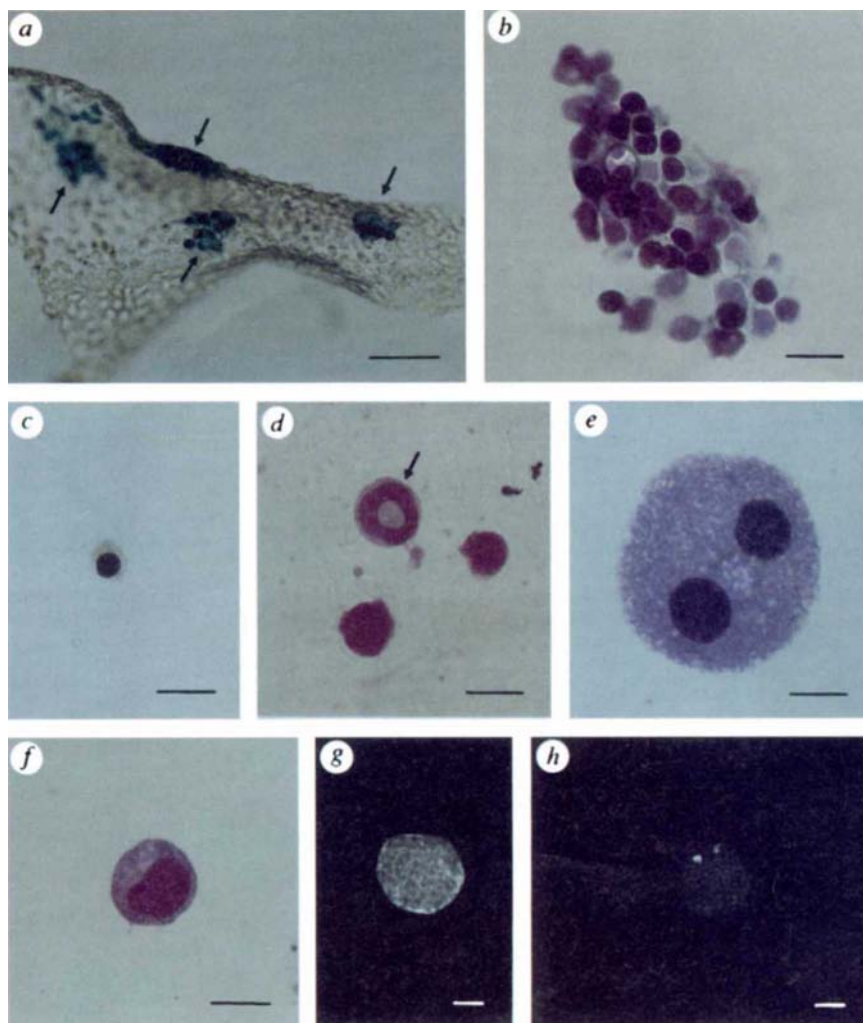
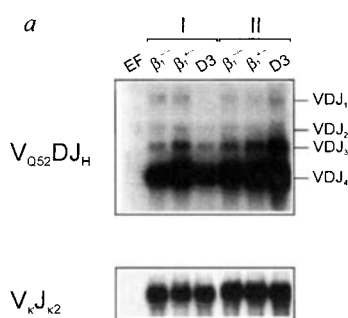


FIG. 4 *In vitro* differentiation of ES cells into B lymphocytes. *a*, Rearrangement of immunoglobulin (Ig) heavy- and light-chain genes during *in vitro* differentiation of ES cells. In two independent experiments, $\beta_1^{-/-}$ and $\beta_1^{-/-}$ ES cells were collected after 20 days of *in vitro* differentiation. Top, products of the polymerase chain reaction (PCR) representing rearrangements of V_{H52} to the four J_H elements (indicated as VDJ_1 to VDJ_4) were visualized after hybridization with a J_H4 -specific internal oligonucleotide. Rearrangement could be



observed for the ES cell clones deficient for either one ($\beta_1^{-/-}$) or both ($\beta_1^{-/-}$) alleles. In all experiments, the parental ES cell line D3 and embryonic fibroblasts (EF) served as positive and negative controls, respectively. Bottom, rearrangements of the Ig light-chain gene were analysed for V_K to J_K2 . All samples from differentiated ES cells were positive. *b*, Frequency of differentiated B cells in cultures of wild-type and $\beta_1^{-/-}$ embryo bodies. The number of B-cell precursors was calculated as the frequency of responding cells in a particular subset from 100 embryo bodies.

METHODS. PCR amplification, gel electrophoresis and blot hybridization of Ig heavy- and light-chain rearrangements from differentiated ES-cell DNA have been described¹⁴. Sequences of the V_{H52} , V_K and J_K2 primers and

Experiment	Phenotype of ES derived cells	Frequency of proliferating lymphocytes	Number of B-cell precursors/100 EB
$\beta_1^{+/+}$ ES cells			
I	Unseparated	<1:2900	<4
	AA4.1+	1: 170	780
	AA4.1+ B220-	1: 360	950
	AA4.1+ B220+	1: 240	460
$\beta_1^{-/-}$ ES cells			
I	Unseparated	<1:4000	<3
	AA4.1+	1: 110	910
	AA4.1+ B220-	1: 140	780
	AA4.1+ B220+	1: 80	250
II	AA4.1+	1: 220	560
	AA4.1+ B220-	1: 180	1340
	AA4.1+ B220+	1: 150	460

probes have been reported^{14,26,27}. Differentiated ES cells were separated after 15 days in culture by magnetic-activated cell sorting with the indicated antibodies. Cells were fractionated according to their cell surface²⁸ and cultured at limiting dilution over adherent layers of mitomycin-C-treated S17 fibroblasts (2,000 cells per well) in 200 μ l Iscove's modified essential medium containing 10% fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol and 100 U ml⁻¹ recombinant murine IL-7 (Boehringer). After 15 days, the presence of B cells was routinely confirmed by subcloning on S17 cells in the presence of IL-7, followed by stimulation with 10 μ g ml⁻¹ lipopolysaccharide (Sigma) for 10 days and measurement of secreted Ig.

We next investigated whether β_1 -null blood cell precursors from the yolk sac were able to enter the embryonic vascular system and colonize the fetal liver. GPI analysis of fetal blood as well as liver from 18 $\beta_1^{-/-}$ $\rightarrow$ WT chimaeric embryos analysed at 13.5, 14.5 and 15.5 days of gestation, respectively, demonstrated that $\beta_1^{-/-}$ cells were present in blood (Fig. 1c) but not in liver (Fig. 1d). To confirm these results, nucleated blood cells and cells from liver of the same chimaeric embryos were cultured in the absence or presence of G418. In control cultures of blood and liver without G418, large numbers of erythroid and myeloid colonies appeared. In the presence of G418, however, colonies formed from blood cells (Fig. 1e) but not from liver cells. In addition, GPI analysis of colonies obtained after culture of fetal liver cells without G418 never showed a 129 GPI isoform (Fig. 1e), thus confirming the absence of β_1 -null cells from liver. These results indicate that β_1 integrins are essential for colonization of fetal liver by haematopoietic precursor cells.

The finding is unexpected that the lack of β_1 integrin does not affect blood cell differentiation but inhibits homing to the fetal liver. None of the naturally occurring or experimentally induced mutations in genes encoding cell-adhesion receptors interferes so drastically in blood cell migration^{15–19}. Several members of the β_1 integrin subfamily play a role in migration, which includes both adhesion to endothelial cells as well as binding to the basement membrane²⁰. To identify the α -subunit(s) responsible for the phenotype described, it will be necessary to investigate further ES cell lines carrying specific mutation in these genes. □

Received 15 September 1995; accepted 22 January 1996.

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ACKNOWLEDGEMENTS. We thank F. Alt and H. Mossmann for RAG-2-deficient mice, K. Imai for discussion, and C. Linington for critically reading the manuscript. E.H. is on a leave of absence from the Dipartimento di Genetica, Biologia e Chimica Medica, University of Torino, Italy. The work was supported by a grant from the Deutsche Forschungsgemeinschaft and the Hermann and Lilly Schilling Stiftung (to R.F.).

Selection of RNA-binding peptides *in vivo*

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MANY principles of sequence-specific DNA recognition have been established over the past decade, largely from structural studies of protein–DNA and drug–DNA complexes. On the basis of these principles, it has been possible to design or select variants of known structural motifs, including zinc-fingers^{1–3} and minor groove-binding drugs⁴, that bind desired sequences. Here we describe a strategy, based on transcriptional termination in bacteria, to identify specific RNA-binding peptides using the arginine-rich RNA-binding motif⁵ as a framework. Peptides were isolated from two combinatorial libraries that bind tightly and specifically to the Rev response element of HIV. It appears that α -helical peptides resembling Rev were selected from one library whereas new peptides that probably do not form helices were selected from the other, suggesting that the arginine-rich motif may be a particularly versatile framework for recognizing RNA structures.

Previous studies with arginine-rich model peptides from HIV Tat^{6–8}, HIV Rev^{9,10}, BIV Tat^{11,12}, and bacteriophages λ and P22 N proteins¹³ have suggested that specific, high-affinity RNA binding can be achieved without a large protein framework and with a relatively limited set of amino acids (Fig. 1a). The Rev and N peptides bind to their RNA sites in α -helical conformations^{10,13}, the BIV Tat peptide binds as a β -hairpin^{12,14}, and the HIV Tat peptide may bind in an extended conformation.¹³ Except for an isoleucine in BIV Tat¹², hydrophobic amino acids are generally not involved in the interactions, allowing us to construct combinatorial libraries expected to encode RNA-binding peptides using only hydrophilic or charged amino acids. Oligonucleotides encoding peptide libraries were fused to the bacteriophage λ N antiterminator protein and a two-plasmid β -galactosidase reporter

system¹⁵ (Fig. 1b) was used to identify interactions with RNA hairpins of interest.

To test the system, the 19-amino-acid amino-terminal RNA-binding domain of N was replaced by arginine-rich peptides from HIV Rev, BIV Tat, or HIV Tat, and the box B hairpin was replaced by HIV Rev response element (RRE), BIV TAR, or HIV TAR. Antitermination was observed only with specific peptide–RNA interactions (Fig. 2a). The activities of HIV Rev and BIV Tat–N fusion proteins on their respective reporters were lower than wild-type N on the nut reporter, perhaps because the box B hairpin contributes additional interactions to the antitermination complex^{16,17}, but activities were well above background levels. The lack of activity of the HIV Tat–N fusion protein on the HIV TAR reporter was expected because additional cellular factors are needed for high-affinity binding¹⁸.

We examined the ability of the system to distinguish between different RNA-binding affinities by creating a small BIV Tat peptide library in which the codon for isoleucine at position 79 was randomized. The hydrophobicity of this amino acid is important for binding to BIV TAR¹². The library was screened on the BIV TAR reporter and plasmids were sequenced from 75 colonies with varying blue intensities. All 15 dark blue (+++) colonies encoded large hydrophobic residues (I, Y, F and L), 29 medium blue (++) colonies encoded smaller hydrophobic or uncharged residues, and 31 light blue (+) or background (–) colonies generally encoded amino acids with charged or small side chains. Antitermination activities determined by β -galactosidase assays correlated well with activities measured by CAT assays in mammalian cells (Fig. 2b).

We next asked whether we could identify specific RRE-binding peptides from combinatorial libraries. The Rev peptide (TRQARRRRRRWR) requires six amino acids (indicated in bold) and a stable α -helical structure for specific RRE recognition^{10,19}. We wished to determine whether there are other ways to recognize the RRE, and whether alternative peptides would bind as tightly, or even more tightly, than Rev. We constructed a 14-residue (14-mer) peptide library consisting of arginine, serine, asparagine or histidine at each position (RSNH library), and a codon bias toward arginine. The mixture of 4¹⁴ ($\sim 2.7 \times 10^8$) sequences was expected to contain 'Rev-like'

TABLE 1. Antitermination activities of the HIV Rev peptide and selected clones

	Sequence	RRE		RRE ⁻		BIV TAR	
		X-gal	ONPG	X-gal	ONPG	X-gal	ONPG
Rev	TRQARRNRRRRWRR	++ +	66	—	7.5	—	6.6
Rev (S34)	SRQARRNRRRRWRR	++ +	50	—	6.8	—	6.9
BIV Tat		—	1.4	—	1.2	++ +	35
RSNH-1	SRSSRRNRRRRRRR	++ +	67	—	8.8	—	8.7
RSNH-2	SRSRRRNRRRRRRR	++	54	—	13	—	9.5
RSNH-3	NHRRRRRHRRRRRR	+	3.5	—	3.2	—	2.7
RSNH-4	NHRRRRRQRRRRRR	++	12	—	2.5	—	3.1
RSNH-3 (H2S)	NSRRRRRHRRRRRR	—	2.3	—	2.4	—	2.5
RSNH-4 (H2S)	NSRRRRRQRRRRRR	+	7.1	—	2.4	—	1.7
RSNH-4 (H2R)	NRRRRRRQRRRRRR	—	1.4	—	1.0	—	1.1
RSNH-4 (Q8N)	NHRRRRRNRRRRRR	—	2.7	—	2.8	—	2.4
RSNH-4 (N1R)	RHRRRRRQRRRRRR	—	1.4	—	1.0	—	1.2
Rev	TRQARRNRRRRWRR	++ +	66	—	7.5	—	8.7
BIV Tat		—	2.8	—	3.4	++ +	37
RSG-1	RCRRRR RGSR SGASRRRRR	++	14	—	2.5	—	3.2
RSG-2	SPCR SR SG SSRR RRRRR	++ +	39	—	5.7	—	6.5
RSG-3	RRRRR SSGS GASRRRRR	++	14	—	5.8	—	5.2
RSG-1 (C2R)	RRRRRRGSR RRSGAS RRRRR	++	9.6	—	2.7	—	2.8
RSG-1 (A13S)	RCRRRRGSR RRSGS RRRRR	+	8.2	—	3.4	—	4.1
RSG-1 (R10A)	RCRRRRGSRASGASRRRRR	+	7.1	—	2.4	—	3.2
RSG-1 (R10P)	RCRRRRGSR P SGASRRRRR	++	22	—	3.3	—	4.4

Results in top part are from the RSNH library and in bottom part from the RSG library. The RRE⁻ reporter contains a G46 : C74 to C : G base pair substitution that markedly reduces Rev peptide binding affinity¹⁰. Bold amino acids in Rev are important for binding and analogous residues in the Rev-like RSNH-1 and RSNH-2 are indicated. Mutations in the non-Rev-like RSNH-3 and RSNH-4, and in RSG-1 are underlined. RSG-2 and RSG-3 containing single codon deletions. The RSNH library was constructed with a degenerate oligonucleotide, 5'-AGGAGAATCCCCATGGCC(XYT)₁₄GCAGCTGCGGCGAATGCAGCAAAATCCGCTG-3', where X is a C : A mixture at a 3 : 1 ratio and Y is an A : G mixture at a 1 : 3 ratio. Each randomized codon (XYT) encodes R : S, H : N at a ratio of 56.25% : 18.75% : 6.25%. The RSG library was constructed with a degenerate oligonucleotide, 5'-AGGAGAATCCCCATGGCCCGTGCCTGCGCGTGCCTG(XGY)₉CGTGCCTGCGCGTGCAGCTGCGGCGAATGCAGCAAAATCCCCTG-3', where X is a C : A : G mixture at a 1 : 1 : 1 ratio and Y is a T : C mixture at a 1 : 1 ratio. A primer (5'-CAGGGGATTGTGCTGCATTC-3') was annealed to the oligonucleotides and double-stranded DNAs were synthesized using Sequenase 2.0 (USB). The RSG library was designed to encode nine randomized positions (19,683 sequences) flanked on each side by five arginines. Most of the arginine-rich RNA-binding peptides studied to date can be encoded by nine amino acids of appropriate sequence if presented in an arginine context (Fig. 1a), and the low complexity of the RSG library allowed it to be exhaustively screened. DNAs for each library were ligated into the *BsmI* and *NcoI* sites of pBRepTacN* and RRE-containing reporter cells were transformed with the ligation mixtures. For the RSNH library, ~600,000 colonies were screened and 1,920 visibly blue colonies were picked. For the RSG library, ~200,000 colonies were screened and 425 blue colonies were picked. To eliminate false-positives, N-expressing plasmids were purified from pooled blue colonies and were rescreened with RRE reporter cells. False-positives were observed at a frequency of ~0.2–0.5%, similar to that observed spontaneously with the reporter cells alone. Plasmids from individual blue colonies were purified and screened with RRE and BIV TAR reporter cells to identify N-fusion proteins specific for the RRE. The majority (~85% for the RSNH library and ~50% for the RSG library) of the N-fusion plasmids exhibited nonspecific antitermination activity, showing at least some activity on both reporters. We sequenced 19 RRE-specific clones from the RSNH screen and four unique sequences were found. We sequenced 33 RRE-specific clones from the RSG screen and three unique sequences were found. In a tertiary screen designed to eliminate false-positives arising from mutations outside the cloned peptide region in the N-expressing plasmids, oligonucleotides corresponding to the selected positives were synthesized, recloned, and plasmids were retested with RRE and BIV TAR reporter cells. All showed RRE-specific activity.

peptides with the consensus sequence SRxxRRNxxxRxxx (the T-to-S mutant in wild-type Rev is fully active; Table 1). The RSNH library was transformed into cells containing the RRE reporter, ~600,000 colonies (~0.2% of the total library) were screened, false-positives were eliminated, and four unique sequences were identified that antiterminate through the RRE (Table 1). Two of the four selected peptides (RSNH-1 and RSNH-2) were Rev-like and exhibited specific antitermination activities comparable to the wild-type Rev peptide (Table 1). The two remaining peptides (RSNH-3 and RSNH-4) did not match the Rev consensus sequence, and RSNH-4 contained a glutamine residue that apparently arose from mutation of a histidine codon. The two non-Rev-like peptides exhibited weaker but specific antitermination activities (Table 1). The activity of RSNH-3 was clearly detectable only using the colony colour assay.

Because the spacing of non-arginine residues in the two non-Rev-like peptides was similar to the spacing of serine and asparagine in Rev (RSNH-3 and RSNH-4 have histidine at position 2 and either histidine or glutamine at position 8), we tested the activities of several mutants to assess whether the mode of binding might be related to Rev. The identities of the non-arginine side chains were found to be important for binding (Table 1) and do not match the side-chain requirements in Rev.

To confirm that the antitermination activities measured *in vivo*

accurately reflected RNA-binding properties of the peptides, we measured binding affinities and specificities of corresponding synthetic peptides *in vitro*. The results correlated well with the antitermination assay: the two Rev-like peptides bound the RRE with affinities comparable to the wild-type Rev peptide, whereas the RSNH-3 and RSNH-4 peptides bound with lower affinities (Fig. 3*a, e*). All peptides bound with low affinity to three mutant RREs known to bind Rev weakly¹⁰ (Fig. 3*a, e*), suggesting that similar binding sites may be recognized although discrimination by the non-Rev-like peptides is poor. Circular dichroism was used to assess whether the selected peptides adopted α -helical conformations (Fig. 3*b*). The 14-amino-acid wild-type Rev peptide was less helical than a previously studied 17-amino-acid version (11% versus 43%), and the selected Rev-like peptides were even less helical (5–6%). The non-Rev-like peptides showed 1–2% helix formation, probably explaining the marginal binding specificities for the RRE *in vitro*. We suspect that the non-Rev-like peptides may be more helical in the context of the N-fusion proteins and therefore have some specific antitermination activity *in vivo*.

To identify peptides that might bind the RRE in non-helical conformations, we constructed a second library consisting only of arginine, serine and glycine residues (RSG library), anticipating that glycine-containing peptides would be unlikely to form stable α -helices. We exhaustively screened a library having nine ran-

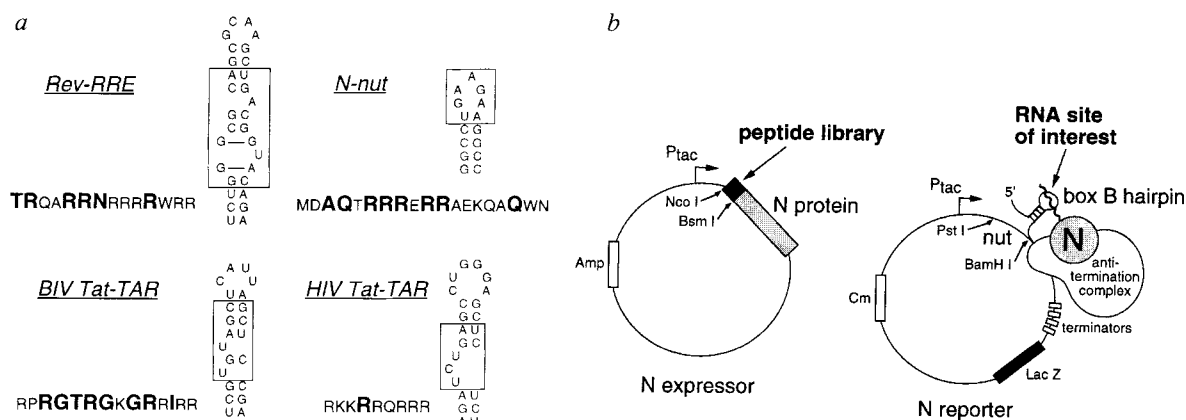
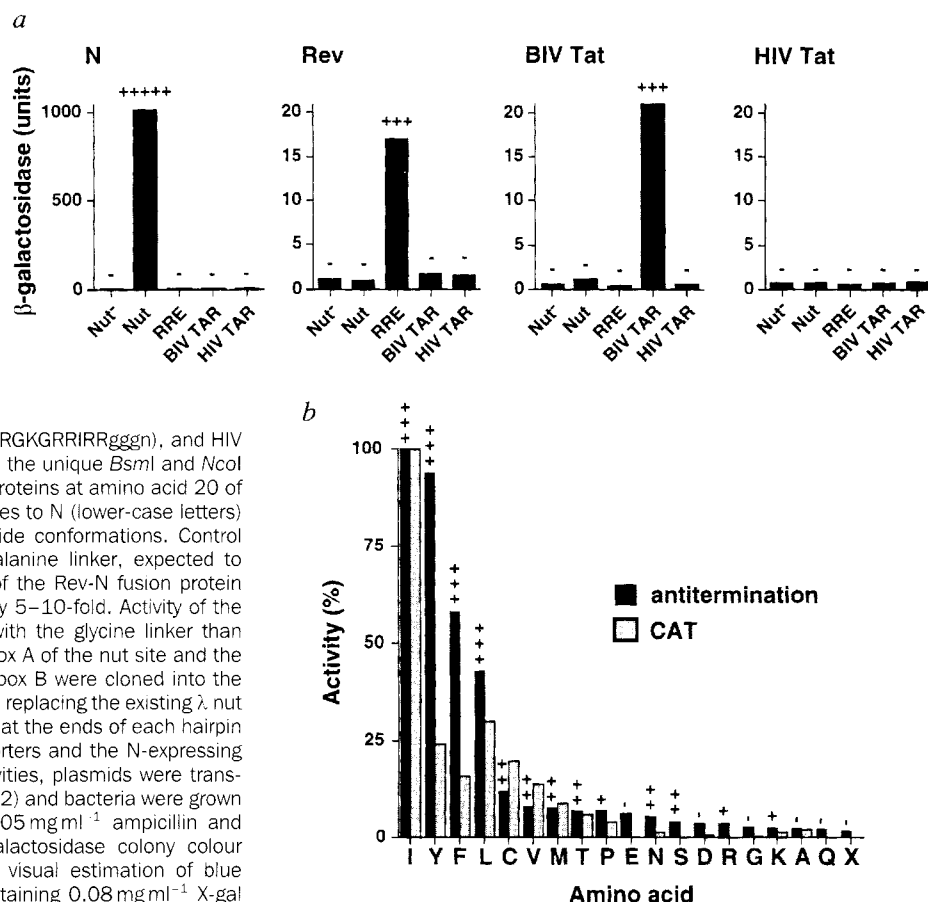


FIG. 1 **a**, Arginine-rich peptides and their specific RNA-binding sites: HIV Rev₃₄₋₄₇ (E47 → R) and RRE IIB; λ N₁₋₁₉ and box B of the nut site; BIV Tat₆₈₋₈₁ and BIV TAR; HIV Tat₄₉₋₅₇ and HIV TAR. The HIV Rev and λ N peptides must be in α -helical conformations to bind specifically to their RNA sites^{10,13}. The Rev peptide shown is shorter than the previously studied Rev₃₄₋₅₀ version and has a single substitution that maintains the overall charge of the peptide; both peptides give similar activities in the assays described in this paper. Amino acids important for binding are indicated in bold and binding sites in the RNAs are boxed. Important amino acids in λ N are tentatively assigned from mutagenesis of the intact protein¹⁵. **b**, The two-plasmid system for measuring transcriptional antitermination by the λ N

protein¹⁵. The λ N protein binds to the box B hairpin of the N-utilization (nut) site on the nascent RNA transcript. N, along with other host factors, forms an antitermination complex that allows RNA polymerase to transcribe through termination sites²¹. In the two-plasmid system¹⁵, N protein is expressed under the control of a tac promoter on a pBR322-derived plasmid, and β -galactosidase, also under control of a tac promoter, is expressed on a pACYC-derived reporter plasmid, with nut and termination sites upstream of *lacZ*. Because the interaction of N with the box B hairpin is itself mediated by an arginine-rich peptide (shown in **a**), it was anticipated that the interaction may be replaced by other arginine-rich peptide-RNA interactions.

FIG. 2 **a**, Antitermination by N proteins fused to heterologous arginine-rich peptides. Activities of N and N-fusion proteins were measured using the reporters indicated. β -Galactosidase activities were determined by ONPG assays (units) and by X-gal colony colour assays (+, -). **b**, Antitermination activities of BIV Tat I₇₉ mutants on the BIV TAR reporter. β -Galactosidase activities determined by the ONPG assay are expressed as per cent of wild-type BIV Tat peptide activity and are plotted next to activities previously determined in HeLa cells using an HIV LTR-CAT reporter²². Activities determined by X-gal assays also are shown (+, -).

METHODS. Synthetic oligonucleotide cassettes encoding HIV Rev (maTR-QARRRRRRRRWRaaaaan), BIV Tat (mgRPRGTRGKGRIRRRgggn), and HIV Tat (mRKKRRQRRR) peptides were cloned into the unique *BsmI* and *NcoI* sites of pBRptacN* (ref. 15), creating fusion proteins at amino acid 20 of the λ N protein. Amino acids linking the peptides to N (lower-case letters) were chosen to help stabilize particular peptide conformations. Control experiments (not shown) indicated that an alanine linker, expected to stabilize an α -helix, roughly doubled activity of the Rev-N fusion protein whereas addition of glycines decreased activity 5–10-fold. Activity of the BIV Tat-N-fusion protein was slightly higher with the glycine linker than without a linker. Oligonucleotides containing box A of the nut site and the appropriate RNA hairpin (Fig. 1a) in place of box B were cloned into the unique *PstI* and *BamHI* sites of pACnutTAT13¹⁵, replacing the existing λ nut site. Two additional GC base pairs were added at the ends of each hairpin stem. Nut and Nut⁻ (a nut site deletion) reporters and the N-expressing plasmid are described in ref. 15. To test activities, plasmids were transformed into *Escherichia coli* strain N567 (ref. 22) and bacteria were grown on tryptone plates or in broth containing 0.05 mg ml⁻¹ ampicillin and 0.015 mg ml⁻¹ chloramphenicol. In the β -galactosidase colony colour assay, the number of plus signs represents visual estimation of blue intensity after growing colonies on plates containing 0.08 mg ml⁻¹ X-gal and 0.024 mg ml⁻¹ IPTG (to induce the tac promoters) for ~48 h at 34 °C. β -Galactosidase activity was measured in permeabilized cells using an ONPG colorimetric assay^{15,23}. The activity measured on plates and in permeabilized cells correlates roughly as follows: + + + + +, >200 units β -galactosidase/1 OD₆₀₀ unit cells; + + + +, 100–200 units; + + + 10–100 units; + +, 2–10 units; +, 1–10 units; -, background. At low activities, the colony colour assay appears to be more sensitive than the ONPG assay, presumably because colony colour reflects β -galactosidase



activity accumulated during 48 h of growth. The level of activity in the ONPG assay reflects activity after 1 h of induction and depends on the rate of N-fusion protein synthesis, which may differ significantly between clones. Western analyses using anti-N polyclonal antiserum (kindly provided by N. Franklin, University of Utah) indicated that steady-state expression levels of each N-fusion protein were slightly below that of wild-type N (data not shown).

domized positions flanked by arginines, and identified three unique sequences that specifically antiterminated through the RRE (Table 1). All three contained a consensus sequence (SxxSGxSRRRRR) but also contained mutations not present in the original library, suggesting that the designed RSG library does not encode RRE-binding peptides. RSG-1 contained a cysteine mutation in the flanking region and an alanine in the randomized region. Replacing cysteine with arginine (C2R) had little effect on activity, whereas replacing alanine with serine (A13S) reduced activity in the colony colour assay (Table 1). RSG-1, RSG-1(C2R), and RSG-2 peptides were synthesized and RRE-binding affinities were measured. All three peptides bound with affinities similar to the Rev peptide, and RSG-2 bound with even higher specificity

relative to three mutant controls (Fig 3c, e). Circular dichroism spectra of RSG-1, RSG-1(C2R), and RSG-2 peptides indicated no detectable helix formation (Fig. 3d), and introducing a proline at position 10 of RSG-1, in the context of the N-fusion protein, did not reduce RRE-specific antitermination (Table 1), further suggesting that the peptide does not require a preformed helical conformation to specifically bind the RRE. Although the RSG peptides appear to bind to the same RNA site as Rev, it seems unlikely that they bind as α -helices or that the details of the interactions are identical. Additional structural studies are required to establish the conformations of the bound peptides, the amino-acid-RNA contacts, and the similarities to the Rev binding site.

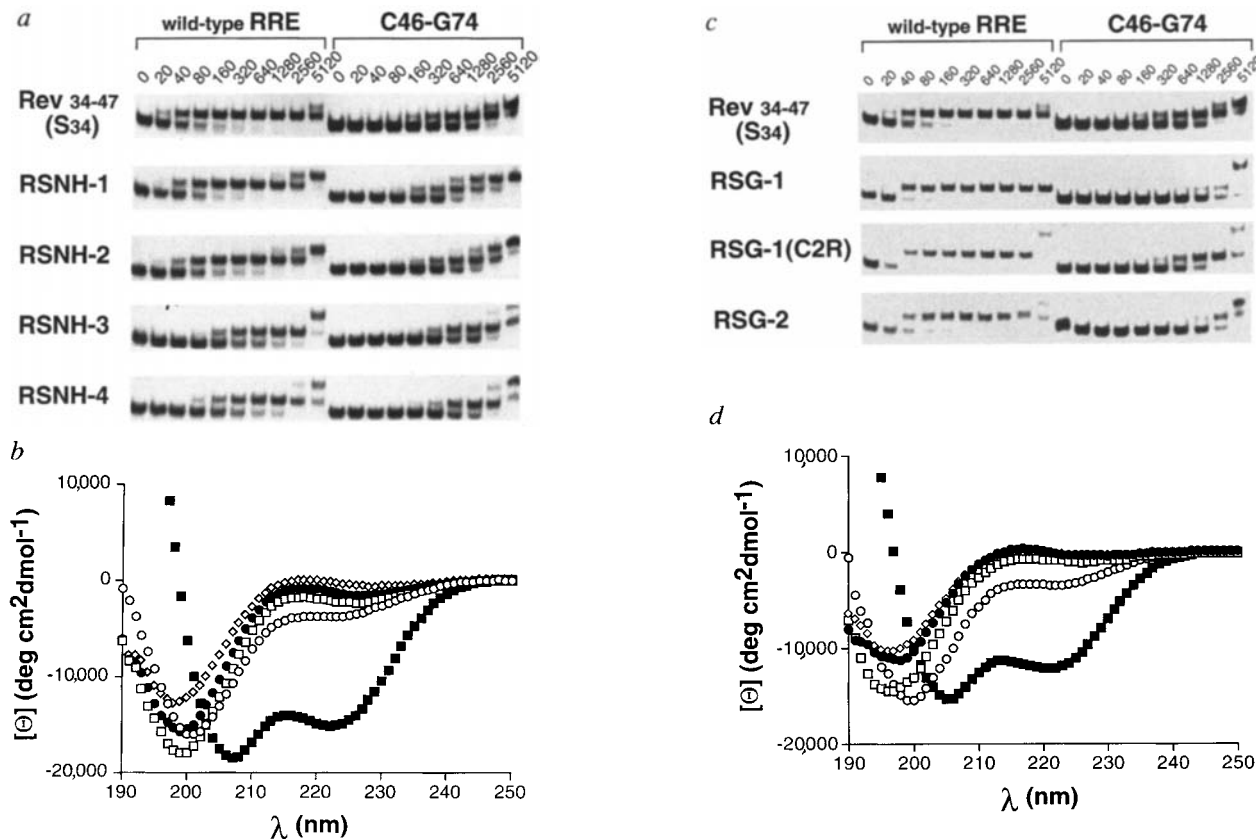


FIG. 3 a, RNA-binding gel shift assay of selected peptides from the RSNH library. Synthetic Rev₃₄₋₄₇ (S₃₄) or selected peptides were bound to wild-type or mutant RRE RNAs at the peptide concentrations indicated (nM). b, Circular dichroism (CD) spectra of Rev₃₄₋₅₀ (■), Rev₃₄₋₄₇ (S₃₄) (○), Rev-like peptide RSNH-1 (□), RSNH-4 (●), and RSNH-3 (◇). c, RNA-binding gel shift assay of selected peptides from the RSG library. d, CD spectra of Rev₃₄₋₅₀ (■), Rev₃₄₋₄₇ (S₃₄) (○), RSG-2 (□), RSG-1 (●), and RSG-1 (C2R) (◇). e, Summary of apparent K_D s (nM) between synthetic peptides and wild-type and mutant RRE RNAs. Note that RSG-1 and RSG-2 bind some of the mutant RREs with lower affinity than does Rev₃₄₋₄₇ (S₃₄) and thus show greater specificity toward the RRE. However, because specific binding by the Rev peptide correlates with α -helix content¹⁰, and because Rev₃₄₋₄₇ (S₃₄) contains only ~11% helix, we expect that the intrinsic affinity of Rev₃₄₋₄₇ (S₃₄) for the RRE is ~10-fold higher than seen in these experiments. All binding constants were determined in the presence of a large excess of competitor tRNA. nd, Not determined. METHODS. Peptides were synthesized on an Applied Biosystems model 432A peptide synthesizer and purified as described¹¹. All peptides were capped by a succinyl group at the N terminus and by four alanines and an amide group at the C terminus. Peptide molecular masses were confirmed by electrospray mass spectrometry (University of Michigan Protein and Carbohydrate Structure Facility). Internally labelled RNAs were prepared using T7 RNA polymerase as described¹¹. RNA-binding gel shift assays were performed by incubating peptide and RNA at 4 °C in 10- μ l binding mixtures containing 10 mM HEPES-KOH, pH 7.5, 100 mM KCl, 1 mM MgCl₂,

	RRE	C46-G74	A47	A71
Rev 34-47 (S ₃₄)	40	600	500	1,000
RSNH-1	50	500	nd	nd
RSNH-2	60	500	nd	nd
RSNH-3	250	500	2,500	2,500
RSNH-4	250	500	2,000	2,000
RSG-1	30	2,000	500	300
RSG-1(C2R)	30	600	nd	nd
RSG-2	30	2,000	2,000	2,000

0.5 mM EDTA, 1 mM dithiothreitol, 50 μ g ml⁻¹ tRNA, and 10% glycerol. To determine relative binding affinities, 1–5 nM radiolabelled RNAs were titrated with peptide, and peptide-RNA complexes were resolved on polyacrylamide gels¹¹. Apparent K_D s are defined as the concentration of peptide required to shift 50% of the free RNA into the complex. In addition to measuring affinities for the mutant RRE shown in a and c, assays were also performed with two additional RRE mutants (A47 and A71) known to decrease affinity for Rev and Rev peptides¹⁰. CD spectra were measured using an Aviv model 62DS spectropolarimeter as described¹⁰. Samples were prepared in 10 mM potassium phosphate buffer, pH 7.5 and 100 mM KF. The cysteine-containing RSG-1 peptide was treated with 1 mM dithiothreitol and diluted into degassed buffer immediately before recording the spectrum. Mean molecular ellipticity was calculated per amino-acid residue and helical content was estimated from the value at 222 nm (ref. 10).

Given the relatively low sequence complexity needed to select specific RNA-binding peptides and the apparent ability to adopt different secondary structures, we speculate that arginine-rich peptides may have evolved early from a predominantly RNA-based world. Studies with HIV Rev and BIV Tat peptides^{12,19,20} indicate that the RNA and peptide structures are both stabilized upon binding, suggesting that the ribonucleopeptide complex, rather than the individual components, may be viewed as a folding domain. Use of an RNA scaffold for peptide folding might have helped a transition to a protein-based world, before the evolution of hydrophobic protein interiors and sophisticated tertiary structures. □

Received 10 November 1995; accepted 12 January 1996.

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ACKNOWLEDGEMENTS. We thank N. Franklin for generously providing N-expressing and nut reporter plasmids, N567 cells, and N polyclonal antibody, and for many helpful discussions. We thank R. Tipton for peptide and oligonucleotide synthesis, D. Campisi for help in characterizing the RSG peptides, and C. Guthrie, A. Johnson, D. Rio and N. Franklin for comments on the manuscript. This work was supported by the Gladstone Institute of Virology and Immunology and by a grant from the NIH.

ERRATUM

Structure and mechanism of DNA topoisomerase II

James M. Berger, Steven J. Gamblin,
Stephen C. Harrison & James C. Wang

Nature **379**, 225–232 (1996)

A PRINTING error caused Figures 3a and 4a of this Article to be transposed. The legends are correct. □

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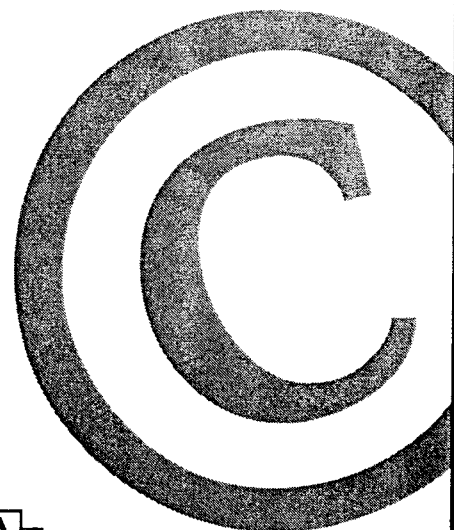
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Gene reading

One-stop shopping for the genetics laboratory — *Drosophila* and mouse modelling systems, a new *in situ* hybridization sealing system, chromosome painting probes and a new family of cell markers for the study of tumour biology.

THE **pfp knockout mouse** from Taconic exhibits a deficiency in perforin, a pore-forming protein stored in CD8⁺ cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. Recent research indicates perforin is a key mediator in CTL and NK cytotoxicity; therefore, the pfp knockout mouse will be a useful tool in immune suppression, transplantation, infectious disease and other immunological research. The deficiency in perforin is caused by the disruption of the *pfp* gene through homologous recombination. The pfp knockout mouse is an addition to the transgenic and knockout rats and mice now available from Taconic, which also offers transgenic breeding services and an exchange programme through its Transgenic Models and Services Division. When this model is combined with the rag-2 knockout model, it creates an improved host for human tumour xenografts or transplants.

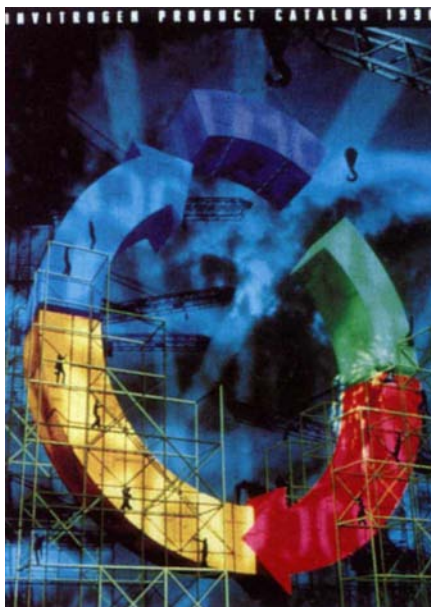
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Exelix is employing two **model systems**, the Pathfinder screen in *Drosophila* and ExCell Trap in mouse to capture the power of genetic homology and uncover gene functions and gene pathways. These technologies can be used to generate animal models of human diseases. In the Pathfinder screen, human disease genes are selected and introduced into the *Drosophila* genome and systematically mutated in order to detect new mutations of genes whose protein products enhance or suppress the gene of interest. These screens selectively reveal the genes whose full expression is crucial for the function of a pathway and encode the key regulatory proteins of the pathway. Genes identified in a Pathfinder screen are cloned and used to develop functional and biochemical assays for drug discovery. ExCell Trap provides information to build disease models, to understand human disease pathways and to identify secretory or transmembrane proteins. All major drugs developed through biotechnology are members of these protein classes.

Reader Enquiry No. 101

New catalogues

The 1996 **catalogue** from Invitrogen contains up-to-date information on more than 350 innovative products for gene expression and analysis. Over 60 new



Invitrogen's new 1996 products catalogue.

products have been added since the 1995 edition. The company's complete line of cloning and expression vectors, nucleic acid purification kits, gene-transfer products, expression systems, PCR-related products and custom services are fully described.

Reader Enquiry No. 102

A new 1996 Ultra Pure Chemical **catalogue** has been released by American Bioanalytical. This new catalogue provides reagents for molecular biology and introduces the new line of Genomic Performance System products for DNA sequencing and DNA purification. The catalogue contains nearly 200 pages of listings, including the newest Ultra Pure agarose offerings.

Reader Enquiry No. 103

Cambio's 1996 **catalogue** is now available. Its 200 pages are packed with thousands of products — both new and familiar — for use in molecular biological research. In addition to Cambio's own range, the catalogue features many exclusive products from Epicentre Technologies, Trevigen, CPG, BioVentures, Janssen and Glen Research. Cambio is now offering chromosome paints, which highlight every human chromosome. A selection of centromeric probes is also available. This new catalogue features

for the first time feline, hamster and murine paints, all sold under the StarFISH trademark.

Reader Enquiry No. 104

Eppendorf now provides an extensive **general catalogue** in English and Spanish, which includes information on all product groups. Liquid handling is side-by-side with clinical and industrial analysis, cell



Multilingual Eppendorf catalogue.

biology and process control. In this new catalogue, product descriptions, technical data and ordering information are supplemented with interesting applications. A catalogue in French is planned for this spring followed by an Italian version in summer.

Reader Enquiry No. 105

Detection

Now available from Porvair Filtronics are two new highly stable **transfer membranes** that are said to offer exceptionally high binding capacities with minimal backgrounds. Together, the PVDF and Magnacharge nylon transfer membranes cover all blotting applications. The nylon transfer membrane is an inherently charged membrane suitable for northern and Southern blotting, DNA fingerprinting and alkaline transfers. It is said to have

high binding capacity (450 $\mu\text{g cm}^{-2}$ DNA) and strength to ensure a high recovery of sample and allow multiple reprobing. The manufacturer states that the membrane has a consistent sensitivity range of 31 μg of total DNA without background interference — essential for DNA fingerprinting. PVDF membranes are directed towards protein blotting applications, being particularly suited to western blots, amino acid analysis and protein sequencing. The PVDF-PLUS transfer membrane has a high protein affinity (average 125 $\mu\text{g cm}^{-2}$ for large proteins) preventing proteins, even as small as 5K, from passing through the matrix. It is suitable for commonly used stains (for example, amido Black, colloidal gold, Coomassie Blue and Ponceau red) and will not degrade, distort or shrink when methanol is used for destaining.

Reader Enquiry No. 106

Using the new SealSure sealing system from Hybaid, reagent loss due to evaporation during *in situ* protocols can now be simply and effectively prevented. This is said to improve the reliability of hybridization and *in situ* gene amplification, which are often adversely affected by changes in salt concentrations brought about by water loss on heating. The system provides a gas-tight seal between standard microscope slides and the system's coverslips at temperatures of up to 97 °C. To accommodate a range of sample dimensions, the seals are available in three sizes to frame areas of up to 1.0 cm², 2.4 cm² and 4.75 cm². A further advantage of this approach is the opportunity to double- or triple-up on the number of samples that can be processed simultaneously on the same glass slide. The frame seal is simply located around the sample on its slide, the reagents are pipetted onto the coverslip and the two are pressed together. After treatment, the coverslip is removed and the sample is ready for analysis. Packaged under 'clean-room' conditions, the product can be sterilized by ultraviolet irradiation and

autoclaving to ensure no artefacts are introduced into cycling procedures.

Reader Enquiry No. 107

Biological Detection offers new software for automated comparative genomic hybridization (CGH) analysis. MultiFluor CGH is a Windows-based software package for analysing digitized images of CGH metaphase spreads for gains or losses of genetic material. It automatically calculates fluorescence CGH ratios for all chromosomes in a metaphase spread(s), then presents the results graphically for detailed assessment. The program



Windows-based software for CGH.

chooses the appropriate chromosomes using built-in criteria, accurately calculates the central axis of each chromosome out to the telomeres, performs local background corrections, normalizes intensity measurements and standardizes chromosome lengths for comparison of data from multiple metaphase spreads. Following analysis, chromosome images are arranged in the CGH-karyotype window according to length. Auto-ID, banding enhancement and other tools are provided for accurate chromosome identification. The program then averages the ratios of all homologous chromosomes to provide statistically meaningful profiles.

Reader Enquiry No. 108

Vysis has released a complete line of reagents for performing comparative genomic hybridization (CGH) experiments. These CGH reagents have been optimized for use with the Quips CGH genetics workstation. CGH is a tool that helps streamline the aberrant DNA search process, showing chromosome abnormalities related to genetic gain or loss. Potentially providing both diagnostic and prognostic utility, the process is expected to accelerate the acquisition of genes critical to cancer and other genetically linked diseases. The new CGH reagent line includes fluorescent-labelled dUTP for labelling test and control DNA, labelling reagents for incorporating fluorescently labelled dUTP into DNA by nick translation, fluorescent-labelled reference DNA, control DNA, hybridization reagents, human Cot-1 blocking DNA and normal metaphase slides. CGH serves as a powerful tool for *in situ* hybridization of whole genomic DNA, using a complete coloured karyotype of the target genome. Its analysis results in a ratio profile of two fluorescent signals whose relative strengths indicate chromosomal subregions that have gains or losses. The cumulative averaging of ratio profiles over a number of metaphase images can improve the performance of these measurements by reducing signal noise. CGH requires a high level of inter- and intra-assay reproducibility to obtain optimum results.

Reader Enquiry No. 109

A new assay for the measurement of leptin, the gene product of the obesity gene, is available from Biogenesis. Recent research has shown that leptin, a 16K plasma protein, may play an important role in regulating body weight by signalling the size of the adipose tissue mass. Levels of leptin are highly correlated with body-mass index — weight loss (from food restriction) is associated with a decrease in plasma leptin levels.

Reader Enquiry No. 110

Oncor has announced the release of an update to its FISH/CGH software application. It includes algorithms for computer-assisted FISH (fluorescent *in situ* hybridization) and CGH (comparative genomic hybridization) analyses. This application is intended to provide users with new insights into their results in an easier-to-use format. Features added to this release include accumulation of data from multiple metaphases, CGH normalization through full image characterization, with local background subtraction on a per-chromosome basis. The software can also process the entire metaphase at once, rather than individually utilizing hand or manual segmentation of each chromosome. The software also features

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an ideogram display that can be utilized to help in identifying specific regions on a chromosome of interest.

Reader Enquiry No. 111

Scotlab Bioscience has recently introduced the Biovation line of **whole chromosome painting probes**. These are produced to uniformly label specific chromosomes, including the telomeric regions. Metaphase cells can be probed directly with these fluorescent probes with hybridization times as short as 2 h, to give clear labelling of translocations and other chromosomal abnormalities.



ScotLab's paint probes include telomeres.

The Biovation painting probes can be supplied as a full set for the labelling of any chromosome, as individual probes or as any desired combination. In each case, probes are supplied ready to use along with hybridization buffer and a full protocol.

Reader Enquiry No. 112

Cancer research

BioWhittaker has introduced a new family of **cell markers for the study of tumour biology**. These seven CD44 variants are said to be sensitive and highly specific marker antibodies for the study of tumour development. They represent a polymorphic family of cell-surface proteins that are expressed on numerous cell types, including monocytes, neutrophils, B and T cells, and fibroblasts. The smallest isoform, CD44std (standard), is derived from ten different exons. Differential splicing of variant exons (v1 to v10) in a number of combinations results in larger CD44 proteins with extended extracellular portions, commonly known as CD44 variants. As CD44std is widely distributed in human lymphoid and non-lymphoid tissues, the variant isoforms are highly specific in their tissue distribution. According to the manufacturer, studies have confirmed that variant CD44 expression correlates with tumour progression and may play an important role in the formation of metas-

tases in humans. The full range of marker antibodies from Bender MedSystems includes anti-CD44var (v5), anti-CD44var (v6), anti-CD44var (v7-v8), anti-CD44var (v10) and anti-CD44var (v3-v10).

Reader Enquiry No. 113

Miltenyi Biotec now offers the new **carcinoma cell enrichment kit**, which uses magnetic cell-separation technology. The new kit allows the efficient and rapid enrichment of epithelial tumour cells from peripheral blood. Carcinoma cells, with an incidence of about one out of 10^6 leukocytes, can be reproducibly enriched to frequencies of about one in 10^3 leukocytes. Epithelial tumour cells circulating in the peripheral blood system differ from normal blood cells by the expression of cytokeratin. After fixation and permeabilization, the tumour cells can be selected using Macs MicroBeads conjugated to an anti-cytokeratin 8/18 antibody. Isolated cells can go straight to flow cytometry, microscopic and molecular analysis.

Reader Enquiry No. 114

Sequencing selection

The new **cycle-sequencing kit**, GATC-BioCycle, is designed to give researchers the freedom to choose a safe and low-cost alternative to radioactive and fluorescent DNA sequencing. The newly developed biotin-labelled dideoxynucleotides allow the use of unlabelled primers, without the need for expensive labelled primers. They are also designed to reduce sequencing errors by eliminating nonspecific chain terminations (full stops). The template-DNA is added to the four GATC-BioCycle sequencing mixes, cycle sequenced with Thermo Sequenase and analysed with the GATC 1500 sequencing system. The kit contains all the required chemicals, controls and protocol. Optimized for use with Thermo Sequenase (not included in the kit), the sequencing kit is designed to ensure reliable, non-radioactive sequencing of PCR products, plasmids, phagemids

or even cosmids. Sequences of PCR fragments are readable up to the last base. Each kit is fully tested for better than 800-nucleotide sequence resolution.

Reader Enquiry No. 115

Boehringer Ingelheim Bioproducts has brought BlueLine to the life science market. Consisting of a full range of **products for gel electrophoresis**, the new series comprises instruments and accessories for all the major electrophoretic applications, including DNA sequencing, DNA agarose submarine gel, vertical slab-gel electrophoresis of proteins and nucleic acids, horizontal isoelectric focusing and electroblotting. This equipment features a modular concept, so that units are compatible with each other.

Reader Enquiry No. 116

National Biosciences (NBI) announced the release of its **custom DNA sequencing services specification sheet**. In order to match the customer's critical scientific requirements and budget, this detailed DNA-sequencing specification sheet defines NBI's broad range of accurate and timely sequencing options. Because of the range of services and expertise available in-house, along with NBI's high-volume synthesis laboratory, sequence data can be turned around quickly. Journal-ready service (JRS) is an option available for those researchers who need sequencing data that is formatted and ready for submission to a scientific journal. A complete sequence analysis is provided, which includes translation, restriction enzyme analysis, GenBank/EMBL homology searches, and DNA and protein motif database searches. Sequence accuracy is said to be guaranteed through multiple reads of each primer extension using two different enzymes. Other sequencing services provided are double-strand, single-strand-plus, standard single-strand and primer-extension services. Double-strand services are for customers who need a guaranteed sequence, but not the JRS extras. All compressions are resolved, and

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PRODUCT REVIEW

any disputed regions are resequenced without charge. The single-strand-plus service includes the economy of single-stranded sequencing and depicts the techniques utilized, the results provided and the benefits, along with compression resolution. The standard single-strand service provides the customer with rapid primer walking and fast project turnaround, while keeping within the customer's budget. The primer-extension service is for simple sequence identification.

Reader Enquiry No. 117

The PhorRunnerII sequencing gel system from Jordan Scientific is designed to run high-quality sequencing gels quickly and easily. Their built-in anodized aluminum thermoplates distribute heat evenly, resulting in clean, straight banding patterns across the entire width of the gel. These sequencers can run 42-cm or 62-cm gels, giving the flexibility needed to adapt to changing protocols. The systems come equipped with the Picket Fence sequencing combs, which are shark-toothed combs

designed to last for years. In addition, there is a built-in clamping system, easy-to-drain removable buffer chambers, interlocking safety lids and heavy-duty construction.

Reader Enquiry No. 118

Odds and ends

A complete line of brand-name **exposure cassettes, intensifying screens, and pre-assembled cassettes and intensifying screens** for life science imaging is now available from Sigma-Aldrich Techware. Designed to improve image quality and to shorten exposure time, these products are suitable for use with most popular autoradiography film sizes. Included in the product line are stainless steel exposure cassettes by both Kodak and Sigma-Aldrich. Designed to be light, tight and durable, these rugged cassettes are capable of withstanding temperatures as low as -70°C and feature a convenient lift-up latch for easy opening and closing. Also offered are Du Pont Lightning Plus, Cronex Quanta III, Kodak X-Omatic, and Sigma-Aldrich intensifying screens, as well as Kodak and Sigma-Aldrich exposure cassette intensifying screen sets. A selection of Kodak cardboard X-ray cassettes is also available.

Reader Enquiry No. 119

The new MutaPlax **transgenic λ -DNA packaging system** from Epicentre Technologies is said to package λ -containing DNA from transgenic animal tissues or cell lines with high efficiency. One MutaPlax Extract can yield up to one million or more total plaques in a standard reaction. The protocol consists of three easy steps and requires only one tube of extract per reaction. For *in vivo* mutagenesis assays, the system is compatible with commercially available transgenic rodents and cell lines containing λ -derived sequences. A transgenic DNA control and *Escherichia coli* host cells are included in the kit.

Reader Enquiry No. 120

Medical Systems has released its new Pico injector, the PLI-100. The injector is designed to be a precise system having easy-to-use controls for the delicate *in vitro* fertilization intracytoplasmic sperm injection technique. The system enables independent control of an injection pipette by gently gathering, retailing and



Medical Systems' *in vitro* injector.

delivering a single sperm; a second holding pipette retains an oocyte. The manufacturer states that ease of operation allows inexperienced or experienced users to utilize all aspects of microinjection quickly and economically.

Reader Enquiry No. 121

Autorad ID, a new **biological film identification system** from Fertile Hemispheres, is designed to allow identification information to be transferred quickly, cleanly and permanently onto autoradiographs, thereby reducing the risk of misidentification and eliminating any threat of tampering. The new system, which works with any biological imaging film, uses a simple darkroom identification printer to transfer the image photographically from a specially designed identification card onto the Autorad's film emulsion. The resulting crisp and clear identification mark therefore becomes a permanent part of the autoradiograph. Under safelight conditions, biological imaging film and an electrophoresed blot are placed in an autoradiography cassette as usual. After the film has been exposed (or before, if preferred) identification information is typed or handwritten onto the card, which has been customized with the institution's name and address. A flash of light instantly and permanently transfers the identifying image from the card onto the autoradiograph. After the identification image has been transferred to the film, peeling off the card's backing converts the card into a versatile adhesive filing label.

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These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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